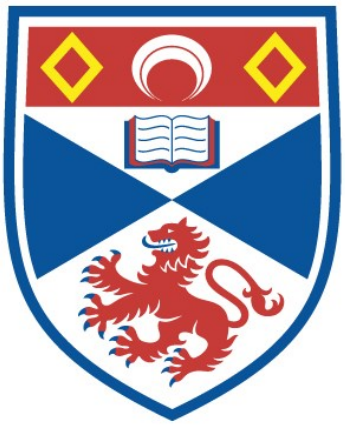
MECHANISMS OF ADENOVIRUS DNA REPLICATION

Alan Monaghan

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Mechanism of Adenovirus DNA replication.

by

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A thesis submitted in partial fulfilment
of the requirements for
the degree of Doctor of Philosophy

School of Medical and Biological Sciences
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ABBREVIATIONS.

A	adenine
A	absorbance (nm)
a.a	amino acid(s)
APS	ammonium persulfate
ATF	adenovirus transcription factor
ATP	adenosine- 5'-triphosphate
ATPγS	adenosine-5'-O-(3-thiotriphosphate)
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CNBr	cyanogen bromide
CMV	cytomegalovirus
cpm	counts per minute
CTP	cytidine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	2'-deoxynucleoside-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
ddATP	2',3'-dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-dideoxycytidine-5'-triphosphate
ddGTP	2',3'-dideoxyguanosine-5'-triphosphate
ddNTP	2',3'-dideoxynucleoside-5'-triphosphate
ddTTP	2',3'-dideoxythymidine-5'-triphosphate
DEAE	diethylaminoethyl
DNase I	deoxyribonuclease I
DNA	deoxyribonucleic acid
DnaA	E.coli initiator protein: binds OriC
DnaB	5' to 3' helicase and activator of primase
DnaC	Complexes with DnaB; delivers DnaB to DNA
ds	double stranded
DTT	dithiothreitol

E.coli	Escherichia coli
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
fmol	femtomole(s)
G	guanine
GTP	guanosine-5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid
HIV-L	human immunodeficiency virus long terminal repeat
HPLC	high performance liquid chromatography
HSV	Herpes simplex virus
HU	non-specific double-stranded DNA binding protein
Ig	immunoglobulin
IHF	sequence -specific ds DNA binding protein; binds to OriC
IPTG	isopropyl-β-*D*-thiogalactopyranoside
ITR	inverted terminal repeat
Kb	kilobases
KDa	1000 dalton molecular weight
LB	Luria broth
LTR	long terminal repeat
MLP	major late promoter
MLTF	major late transcription factor
MMTV	Mouse mammary tumour virus
mRNA	messenger ribonucleic acid
m.u.	map units
M.W.	molecular weight
NF-I	nuclear factor I
NF-I$_{DBD}$	nuclear factor I DNA binding domain
NF-II	nuclear factor II
NF-III	nuclear factor III
NF-III$_{DBD}$	nuclear factor III DNA binding domain
NP40	nonidet P-40

O.D	optical density (nm)
OriC	minimal DNA sequence required for E.coli replication
OriL	Herpes simplex viral origin of replication (long unique)
OriS	Herpes simplex viral origin of replication (short unique)
P53	p53 tumour suppressor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pfu	plaque-forming units
p.i.	post-infection
pH	pondus hydrogen ($-\log_{10}[H^+]$)
PLP	pyridoxal-5'-phosphate
pmoles	picomole(s)
pol	DNA polymerase
pTP	precursor terminal protein
pTP-Pol	precursor terminal protein-DNA polymerase complex
RB	retinoblastoma gene product
rNTP	ribonucleoside-5'-phosphate
RNA	ribonucleic acid
rpm	revolution per minute
SSB	single stranded DNA binding protein
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
SV40	simian virus 40
T	thymine
Tag	T antigen
TBE	Tris-borate-EDTA buffer
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TP	terminal protein
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
ts	temperature sensitive (mutant)

UV	ultra-violet
UTP	uridine-5'-triphosphate
v/v	volume per volume ratio
w/v	weight per volume ratio

ABBREVIATIONS FOR AMINO ACIDS

Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamine	gln	Q
Glutamic acid	glu	E
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	trp	W
Tyrosine	tyr	Y
Valine	val	V

GENETIC CODE

TTT phe F	TCT ser S	TAT tyr Y	TGT cys C
TTC phe F	TCC ser S	TAC tyr Y	TGC cys C
TTA leu L	TCA ser S	TAA OCH Z	TGA OPA Z
TTG leu L	TCG ser S	TAG AMB Z	TGG trp W
CTT leu L	CCT pro P	CAT his H	CGT arg R
CTC leu L	CCC pro P	CAC his H	CGC arg R
CTA leu L	CCA pro P	CAA gln Q	CGA arg R
CTG leu L	CCG pro P	CAG gln Q	CGG arg R
ATT ile I	ACT thr T	AAT asn N	AGT ser S
ATC ile I	ACC thr T	AAC asn N	AGC ser S
ATA ile I	ACA thr T	AAA lys K	AGA arg R
ATG met M	ACG thr T	AAG lys K	AGG arg R

GTT val V	GCT ala A	GAT asp D	GGT gly G
GTC val V	GCC ala A	GAC asp D	GGC gly G
GTA val V	GCA ala A	GAA glu E	GGA gly G
GTG val V	GCG ala A	GAG glu E	GGG gly G

UNITS

^{o}C	degrees Celsius(temperature)
g	gram (mass)
m	metre (length)
mol	mole (quantity)
s	second (time)
Ci	Curie [radioactivity; $3.7x10^{10}$ s^{-1} (disintegrations per second)]
Da	Dalton (relative molecular mass)
F	Faraday (capacitance)
g	gravitational acceleration (9.81 $m.s^{-2}$)
l	litre (volume; 10^{-3} m^3)
M	molar concentration ($mol.l^{-1}$)
min.	minute (time)
S	Svedberg (sedimentation)

ORDER PREFIXES

d	deci	10^{-1}	k	kilo	10^{3}
c	centi	10^{-2}	M	mega	10^{6}
m	milli	10^{-3}	G	giga	10^{9}
μ	micro	10^{-6}	T	tera	10^{12}
n	nano	10^{-9}			
p	pico	10^{-12}			
f	femto	10^{-15}			
a	atto	10^{-18}			

CONTENTS.

Materials and Methods.

RESULTS

ABSTRACT

The development of a cell-free system in which adenovirus DNA synthesis can be initiated in vitro by using the viral genome or plasmids containing the origin of replication as template has led to the identification of the sequences important for origin function and the isolation and purification of the proteins required for viral DNA replication. In vitro studies on adenovirus types 2 and 5 have shown that their replication requires the formation of a large nucleoprotein complex. This is composed of three virally encoded proteins: adenovirus DNA polymerase, precursor terminal protein and DNA binding protein, and two cellular proteins nuclear factor I and nuclear factor III. While the presence of DNA helicases in other eukaryotic DNA replication systems have been well characterised, this was not the case for adenovirus DNA replication. Initial attempts to identify DNA helicase activity associated with any of the adenovirus replication proteins were unsuccessful. However, a novel DNA unwinding activity was found associated with the DNA binding protein (Ad.DBP). We examined the interaction of DBP with partial DNA duplexes and demonstrated that it could displace oligonucleotides annealed to single-stranded M13 DNA. In addition, DBP could also unwind small fragments of fully duplex DNA. Unlike a DNA helicase, DBP promoted DNA unwinding was nucleoside-5'-triphosphate and Mg^{2+} independent and exhibited no directionality. The activity required saturating amounts of DBP and was both efficient and cooperative in nature.The helix-destabilising activity was shown to be situated in the C-terminal domain of the protein. These properties suggest a role for DBP in DNA replication in which DBP destabilises duplex DNA during origin unwinding and replication fork movement.

The second part of the thesis dealt with the characterisation of the putative "active site" of the adenovirus DNA polymerase. This experimental approach was prompted by data from earlier studies which indicated that DBP could increase the processitivity of the polymerase as well as its sensitivity to nucleotide analogue inhibitors. The "active site" was labelled with pyridoxal-5'-phosphate (PLP), a substrate binding site directed reagent for DNA polymerases. Treatment of Ad.5 DNA polymerase with PLP followed by reduction of the enzyme-PLP adduct resulted in irreversible inactivation of the polymerase activity while the 3'-5' exonuclease associated with Ad.5 DNA polymerase was minimally affected. Substrate protection studies indicate that PLP inhibition is complex. Neither template-primer nor substrate dNTP alone showed any protective effect from PLP mediated inhibition. However, the presence of both template -primer and complementary dNTP significantly protected against PLP inhibition. Comparative tryptic mapping of ^{3}H labelled enzyme, modified in the presence and absence of substrates by PLP reaction, on a C-18 reverse phase column, indicated the protection of one peptide from pyridoxylation in the presence of substrates. Amino acid sequence analyses found no sequence to be present in this peak.

1. The Adenoviridae

The Adenoviruses belong to the family Adenoviridae, a group of viruses containing DNA genomes. The adenoviridae family is divided into two genera depending on the host species they infect; Mastadenovirus, whose genus contains 78 or more serotypes, infect a wide range of mammals including cattle, monkeys, sheep, pigs, dogs and humans and Aviadenovirus, which contains 15 or more serotypes which infect a range of birds e.g. chickens, turkeys and ducks (Horwitz, 1990a, 1990b).

Human adenoviruses were first cultured and reported almost simultaneously by Rowe et al (1953) and Hillerman and Werner (1954). Rowe and colleagues discovered the viruses during attempts to establish tissue culture cell lines from tonsils and adenoidal tissues isolated from children with acute respiratory infections. Hillerman's group on the other hand discovered the virus in the same tissue types when carrying out epidemiological studies on U.S. Armed Forces recruits suffering from the same acute respiratory infections as the children in 1953. At present 41 serotypes of adenovirus have been found to infect humans causing acute respiratory, gastrointestinal, ocular and urinary diseases. These viruses are ubiquitous in the human population infecting all ages, races and both sexes equally. Although the majority of adenovirus infections in man are seldom associated with fatal diseases, (the exception being the enteric adenovirus types 40 and 41 which are involved in acute diarrhoeal disease of infants), they are nevertheless considered important human pathogens. Initial studies on the structure and function of adenoviruses were maturated naturally by the result of clinical requirements. However, over the last 40 years their use as a tool in the elucidation of the molecular biology of eukaryotes has been recognised and exploited.

2. Virion Architecture.

The morphology of the adenoviriae virion is highly conserved throughout the genera. The virion consists of two major component structural complexes:- an icosahedral protein shell (capsid) which surrounds an inner core containing the viral DNA and at least two viral structural proteins. Overall the virion contains at least 11 viral proteins ranging from 3KDa-110KDa. Virion size, which is derived from its edge, (the distance between two pentons) was calculated for Adenovirus 5 to be 52nm, giving an icosahedral diameter around the five fold axis of symmetry of approximately 75-88nm (Devaux et al, 1983). A schematic model illustrating the architecture of the adenovirus virion can be seen in figure 1.

2.1 The viral capsid.

The adenovirus capsid consists of 252 capsomeres which are composed of three viral proteins:- 240 hexons (viral protein II) whose organisation dictates the overall icosahedral structure of the capsid and 12 pentons, made up of a base and a fibre (III and IV, respectively), (Valentine and Periera, 1965).

Elucidation of the capsid structure after various treatments such as heating at 56^{o}C, SDS or trypsin or deoxycholate treatments (Russell et al, 1967 ; Laver et al.,1968) revealed that it dissociated into peripentonal hexons which surround each penton and planar 'groups of nine hexons' (GON's). Under an electron microscope these GON's have a propeller like shape and are either left or right-handed depending on whether the inner or outer surface is visualised (Periera and Wrigley, 1974). Physical analysis has shown that the intact hexon is a trimer consisting of three

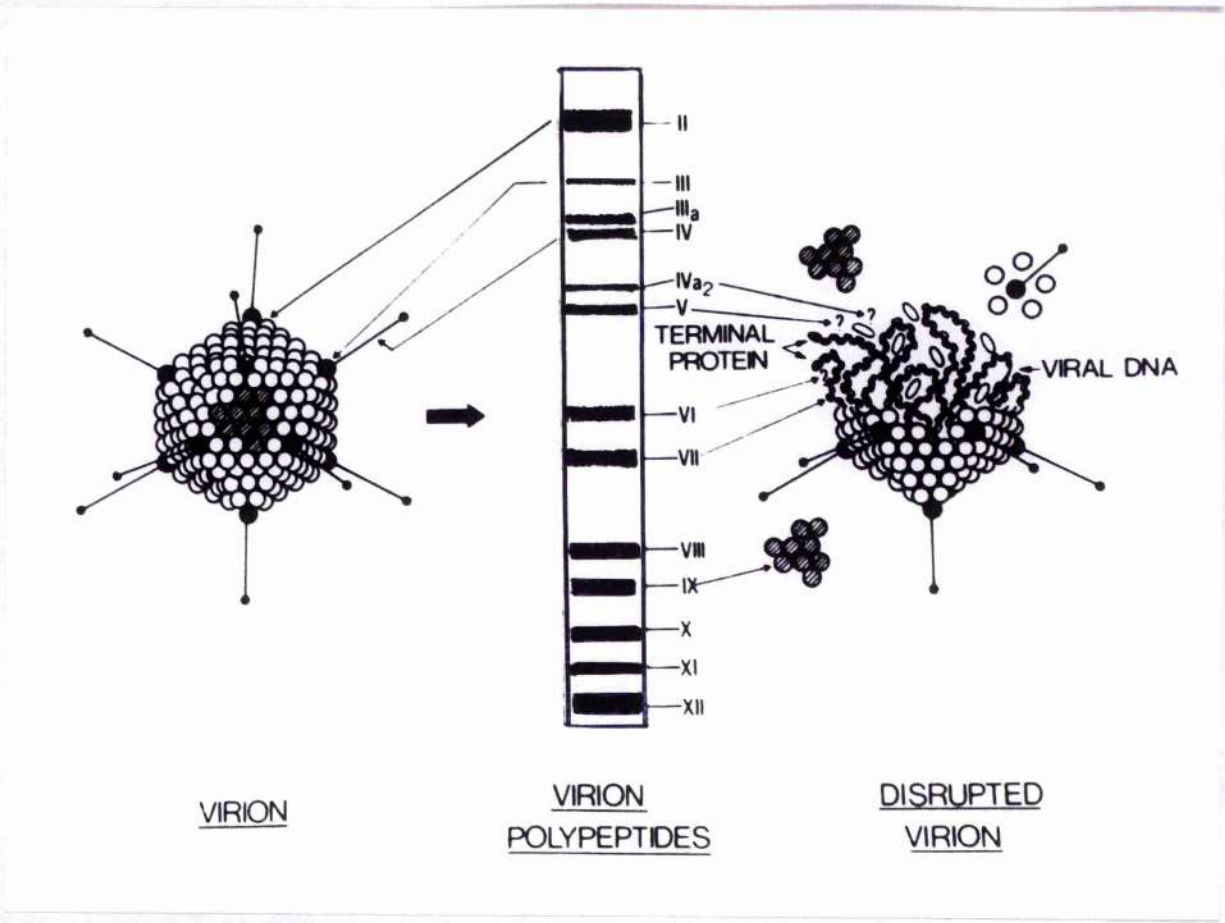


Figure 1. Schematic model of the adenovirus particle.
The SDS-PAGE pattern of the virion polypeptides are shown along with their proposed locations (Russell and Precious, 1982).

identical subunits of 108KDa (Grutter and Franklin, 1974 ; Akusjarvi., et al, 1984).

Through the use of x-ray crystallography, a 3-D structure for the hexon has been obtained and revealed that the hexon trimer consists of two parts; a pseudo-hexagonal base 52A° high with a triangular top 64A° high consisting of three 'towers' (Burnett et al., 1984). Each of the subunits consists of two domains named P1 and P2 which consist of β-barrel structures with loop-out regions between the strands. The three towers situated at the top of the structure, are formed from designated loops 1$_1$ and 1$_4$ from domains P1 and P2 in adjacent subunits and loop 1$_2$ from the third subunit which makes up the trimer (Roberts et al., 1986). This interpretation of the trimeric structure shows that the three subunits of hexon make extensive contacts in both the base and the towers and explains its high resistance to denaturation. Recently, comparative amino acid sequence studies of the predicated sequence of the hexons of Ad.2 and Ad.40 revealed that most of the differences between the serotypes are found in the loops which form the towers, consistent with the fact that the virion surface provides important type specific antigenic determinants (Toogood et al, 1989).

The pentocapsomere consists of a penton base and one or two (avian adenoviruses) thin projections, called fibres. The penton base consists of five subunits of III (85KDa) and the fibre, a trimer of the 62KDa IV protein (Van Oosrum and Burnett, 1985 ; Ruigrok et al., 1990). The fibre usually consists of a shaft 2nm in diameter varying in length between 9-13 nm depending on the serotype and is terminated by a knob-like structure 4nm in diameter. The interaction between the fibre and base are thought to be hydrophobic as they can be easily dissociated by guanidine or deoxycholate (Norrby, 1969).

The capsid structure relies on an additional protein named IX for capsid stabilisation. Studies by Colby and Shenk (1981) demonstrated that an Ad.5 mutant containing deletions in the gene for IX gave rise to virions which were more heat labile and gave individual hexons, not GON's, when dissociated. Further studies revealed that functional IX was essential for the packaging of mature viral genomes during infection (Ghosh-Choudhury et al, 1987). Combined E.M. analysis and x-ray crystallography of GON's and hexon structures, respectively, finally revealed that protein IX acted as capsid "cement", responsible for holding hexons of dissociated virions in the highly stable GON configuration (Furcinitti et al., 1989 ; Stewart et al., 1991 ; Stewart and Burnett, 1993). Viral proteins IIa (66KDa), VI (24KDa) and VIII (13KDa) are also known to be associated with the capsid.

2.2 The viral core.

Disruption of the adenovirus virion by treatment with 10% pyridine or 0.5% deoxycholate removes the coat proteins leaving viral cores (Nermut et al., 1984). These viral cores are composed of viral DNA and four proteins; V, VII , Mu and the terminal protein (Chatterjee et al., 1986). VII which has a molecular weight of 18KDa has been shown ,even after various treatments with urea and acetone, to be closely associated with the viral DNA (Ginsberg, 1979). It is a highly basic protein with approximately 22% of its amino acid sequence as arginine, which is sufficient enough to neutralise the negative charge of the DNA's phosphate backbone. In contrast, the less abundant 45KDa protein V was found to be easily dissociated from the DNA-VII complex, even under relatively mild conditions (Vayda et al, 1983). Protein V has also been shown to be situated adjacent to the hexon, penton and IIIa (Everitt, 1975), possibly forming a shell on the surface of the cores (Brown et al,

1975 ; Nermut, 1979). In addition to V, VII and Mu, other proteins are present in the viral cores at a low copy number. One of these, the terminal protein (TP), is a 55KDa hydrophobic protein, which is covalently linked to the dCMP residue at the 5' end of each viral DNA strand. The role this protein plays in replication will be discussed further in section 6.

The mechanism by which viral proteins and DNA interact and package the genome has been and still is the subject of some controversy. Electron microscopic analysis of cores in various stages of relaxation had shown a "beads on a string" appearance or "rod-like elements" (Mirza and Weber, 1982). This along with micrococcal nuclease treatment data suggested two models for the organisation of the nucleocapsid; the continuous helix model, which proposes that the DNA is wrapped around a single helical filament made up of VII, and the discontinuous "nucleosome" model. Early microscopic analysis clearly favoured the discontinuous model, which proposes that DNA is packaged in nucleosome beads consisting of 6 VII molecules, with two turns of DNA per bead (164b.p.) and a spacer region of 30b.p. between the VII nucleosomes. The 4KDa Mu protein which is even more extremely basic than VIII (54% arginine) has been proposed as an accessory protein, helping VIII maintain the nucleosome structure. However, recent electron microscopic data along with Bal31 endonuclease activity assays on the DNA of subviral particle, has revealed the presence of distinct supercoiled domains within the DNA packaged inside the virion (Wong and Hsu, 1989). The data from these studies have been used to propose a new model in which the viral DNA is held in 8 supercoiled DNA domains (each domain contains 12% of the genome) by interaction with a central core protein, VII. This discontinuous helix model allows the prediction of the positions of the various parts of the genome within such a structure. Interestingly, the E1a and E4a promoters, being at the ends of

the genome, lie just outside the supercoiled regions potentially making them accessible to transcriptional proteins prior to complete dissociation of the virion, suggesting that a topological mechanism may exist for regulation of gene expression. More recently, proof for this theory has come from Wong and Hsu (1990) .They observed the presence of the supercoiled loops during compensatory supercoiling of portions of adenovirus DNA when the DNA double helix was unwound through the intercalation of ethidium bromide. Their observation that the packaging of adenovirus DNA into virus particles was inhibited by topoisomerase II inhibitors suggests that topoisomerase II may be involved in core assembly.

2.3 The Viral genome.

The majority of adenovirus serotypes have a linear, double-stranded DNA genome of approximately 35-36,000 base-pairs (35-36Kb) in length, with the human adenovirus genomes ranging between 34125b.p. in Ad.12 (Sprengel et al., 1994) up to 35,937b.p. for Ad.2 (Roberts et al., 1986). A distinct feature of the adenovirus genome is the presence of a 55KDa protein linked to the ends of the genome. This terminal protein (TP) was shown to be covalently linked to the DNA by virtue of the resistance of the TP-DNA complex to proteolytic digestion, boiling and SDS treatment (Robinson et al, 1973; Sharp et al, 1976; Rekosh et al, 1977). Further studies revealed that both the DNA-protein complex and deproteinised DNA were resistant to digestion with exonuclease VII (5'-3' specificity) and phosphatase and could not be labelled with polynucleotide kinase (adds nucleotides to the 5' ends of DNA). However, they were found to be sensitive to digestion by exonuclease III (3'-5') and labelling (at the 5' end) with terminal transferase (Carusi et al, 1977; Sharp et al, 1976), indicating that TP was attached to the 5' ends of

the genome. The linkage between the TP and DNA was eventually shown to be a phosphodiester bond between the hydroxyl group of serine 625 residue in the TP and the 5' phosphate group of the terminal deoxycytidine of the genomic DNA (Desidero and Kelly, 1981). After the discovery of TP, debate centred on the origin of the terminal protein. Tryptic peptide analysis by Green et al., (1979) demonstrated that the TP's from five different serotypes were similar, suggesting some kind of common cellular origin for the protein. Experimental observations from Rekosh et al., (1981) then indicated that the TP's had different molecular weights, suggesting that TP was in fact a conserved viral protein. Debate continued until Stillman et al., (1981) identified a mRNA between coordinates 11-31.5 on the l-strand of the adenovirus genome, that when translated in vitro gave a protein with a molecular weight of 87KDa. The protein was found to be identical to an 80KDa protein found attached to the 5' ends of in vitro synthesised viral DNA strands. Both the 87KDa and 80KDa proteins were found to be structurally related to the TP, indicating that the TP is synthesised as a precursor molecule (pTP). Further evidence for a precursor molecule came from studies involving a temperature sensitive Ad.2 mutant (Ad.2 ts1). This mutant virus cannot cleave viral precursor proteins to their mature forms at the non-permissive temperature, due to a defective viral protease . When analysed the Ad.2 ts1 virions contained 80KDa, instead of 55KDa TP molecules (Stillman et al, 1981).

Electron microscopic studies on virions treated with 4M Guanidine revealed the existence of single stranded circles of adenovirus DNA (Garon et al., 1972). These circles were found to be thermostable and disappeared after exonuclease III treatment. These observations led to suggestions that the adenovirus contained inverted terminal repeats (ITR's). Early estimates on the size of these ITR's, based on exonuclease

III digestion experiments showed that their size varied from serotype to serotype and put them between 350-1400b.p. long (Garon et al, 1972). However, nucleotide sequence analysis later showed these to be overestimates. The data revealed that the actual ITR size varied from 63b.p. for chicken embryo lethal (CELO) virus (Alestrom et al, 1982) to 165b.p. for Ad.18 (Garon et al, 1982). Sequence analysis data on the ITR's of several serotypes also revealed an asymmetric distribution of G/C and A/T base-pairs. Further investigations on the ITR of Ad.5 revealed that the first 50b.p. of the 103b.p. ITR was composed of 72% A/T residues, whereas the second 50b.p. was only 27% A/T rich (Steenbergh et al., 1977). The ITR's within the human serotypes show a remarkable degree of homology, both Ad.2 and Ad.5 contain identical 103b.p. long ITR's, (Shinigawa and Padmanabhan, 1979), whilst both Ad.7 and Ad.3 have 136b.p. ITR's which differ in only seven positions (Shinigawa and Padmanabhan, 1980).

Within all the human serotype ITR's the sequence 5'-ATAATATACCTTAT-3' from nucleotides 9-22 has been found to be highly conserved (Tolun et al, 1979). This region, contained within the minimal origin of replication (bases 1-18 of the adenovirus origin) is very sensitive to base changes. This conserved domain was found to consist of two regions separated by a region in which single base changes could be tolerated. The latter spans bases 12-16 and any single base change in this region had no effect on in vitro DNA replication levels. However, any base change in the two critical flanking regions had a serious deleterious effect on DNA replication (Temperley et al, 1991; reviewed in Challberg and Kelly, 1989). There are other blocks of high sequence homology within the ITR's of various serotypes which have been shown to bind cellular factors during DNA replication.

Using the nucleotide sequence of the Ad.2 genome which had been determined as 35,937b.p. (Roberts et al, 1986) and data from a variety of techniques, including in vitro transcription/translation, DNA-RNA hybridisations, R-loop and S1 mapping (Weinman et al, 1983), a detailed map of the organisation of the Ad.2 genome was built up. Within the genome are 10 transcription units which are usually described as early or late depending on whether they are transcribed before or after DNA replication has begun. However, due to the complexity of adenoviral transcriptional regulation, the units are now generally referred to as pre-early, early, intermediate and late. The two DNA strands of the genome are classified by the direction of transcription along them; the strand transcribed to the left is termed the l-strand (GC-rich) and the strand transcribed to the right the r-strand (A-T rich), (Bachenheimer et al, 1977). 69% of the viral genes are transcribed from the l-strand. A schematic transcriptional and translational map of the Ad.2 genome, divided into transcriptional units, their mRNA's and the proteins they encode are shown in figure 2 .

3. Adenovirus viral transcription.

Adenoviral transcription from viral promoters is first detected 45 minutes post infection (p.i.) with the viral genes being transcribed from nine initiation sites by the host cell RNA polymerase II (and RNA polymerase III in the case of the viral associated RNA's) in the nucleus of the infected cell.The primary mRNA transcripts are capped at their 5' end with mG 5' pppN and polyadenylated at their 3' end, before entering the cytoplasm where translation takes place and at least 50 viral proteins are synthesised (Philipson et al., 1971). The 5' cap and the 3' tail addition are thought to be important in maintaining mRNA stability and as a signal for the transport of processed nuclear transcripts into the cytoplasm. The

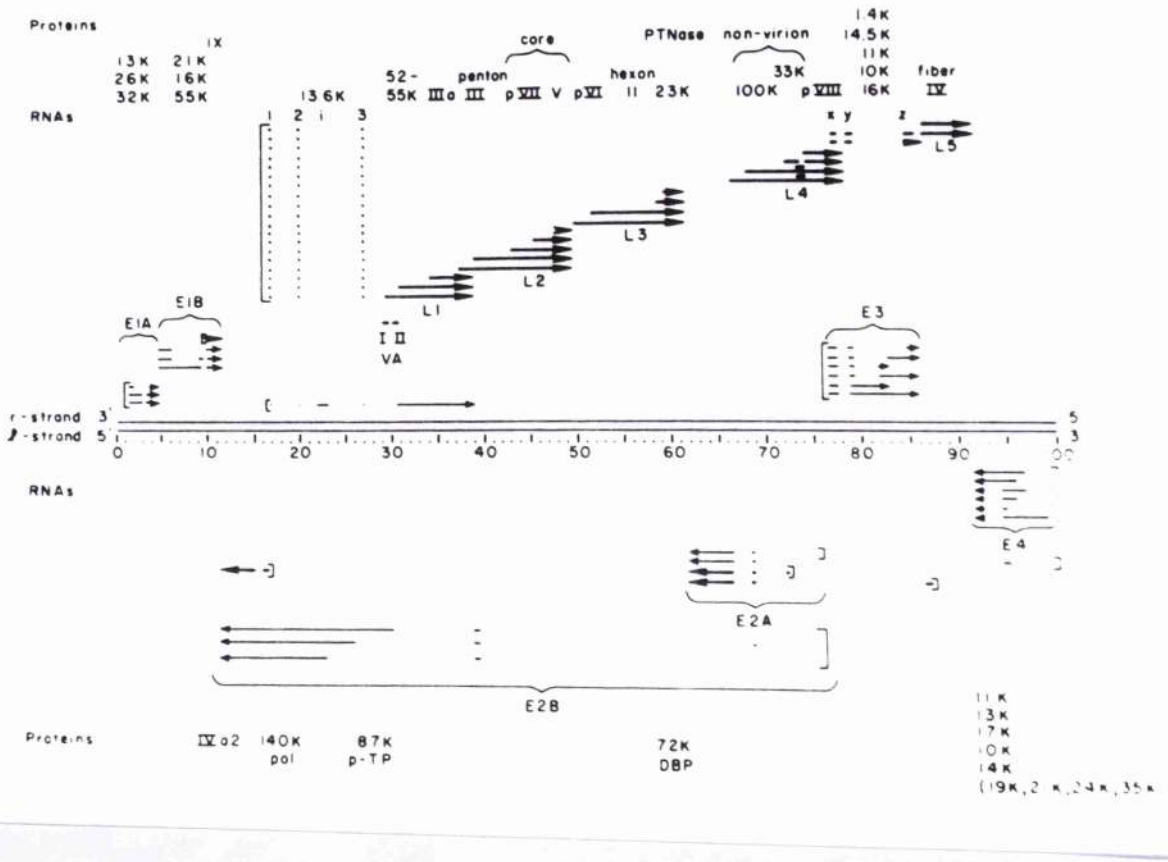


Figure 2. Transcription and Translation map of adenovirus type 2. By convention the r-strand is transcribed from left to right and the l-strand is transcribed from right to left. The viral polypeptides are designated by roman numerals for the virion structural components and in kilodaltons for non-structural virus-coded translation products (Horwitz, 1990a).

regulation of gene expression is fundamental to the progression of the adenovirus lytic cycle and is exerted at a number of levels with transcriptional control being paramount. The order and amounts in which the primary transcripts are synthesised is critical and then each must be processed to give its progeny mRNA's at the appropriate levels. The transportation of the mRNA's out of the nucleus and their stability in the cytoplasm are also subject to differential control (Sharp et al., 1984).

3.1 Early transcription.

The first mRNA transcripts to be detected are those transcribed from the pre-early E1a transcription region (Nevins et al, 1979). E1a transcripts can be detected as early as 45 mins p.i. and usually reach a maximum at 3 hour p.i. The E1a region lies on the r-strand between map units 1.3 and 4.6 and transcribes 3 mRNA's with sedimentation coefficients of 13s, 12s and 9s, respectively. The mRNA's produced share common 5' and 3' ends, differing only in their site of splicing, from which they are produced by separate splicing events from a precursor mRNA (Perricaudet et al., 1979). At early times during infection the predominant mRNA's transcribed from E1a are the 13s and 12s species which give rise to polypeptides of 289 and 243 amino acids, respectively. They differ only in a 46 amino acid internal conserved region (CR3) which is unique to the 289R species (Moran and Mathews, 1987). From nucleotide sequence analysis both the 13s and 12s mRNA were predicted to transcribe proteins with molecular weights of 31.9 and 26.5KDa, respectively (Perricaudet et al, 1979). However, in practice the proteins were found to be heterogeneous, with greater apparent molecular weights than predicted, probably due to various post-translational modifications such as phosphorylation (Yee and Branton, 1985 ; Harlow et al., 1986). The 9s mRNA gives rise to a protein with a molecular weight of 28KDa.

The proteins encoded from the E1a region are involved in both transcriptional regulation and cellular transformation. E1a products have been shown to increase the transcription level of other early viral promoters such as, E1b, E2, E3 and E4, up to 50-fold whilst only increasing its own level 5-fold (Berk et al, 1986; Jones and Shenk, 1979). E1a proteins can also stimulate transcription from cellular promoters such as the heat shock 70 protein, globin promoters (Svesson et al., 1984) as well as viral promoters such as HSVI glycoprotein, thymidine kinase (Everett and Dunlop, 1984) and the HTLVI and II promoters (Chen et al, 1985).

E1a products can also have a negative effect on transcription by repressing the level of transcription from some promoters. E1a has been shown to be involved in the repression of the SV40 and polyoma early promoters (Veliech and Ziff, 1985). The 46R region of E1a which is unique to the 289 amino acid protein has been shown to be vital in transactivation function (Moran and Mathews, 1987). Green et al., (1988) demonstrated that a synthetic peptide comprising the 46R region was sufficient enough to stimulate transcription from several adenovirus promoters. The promoters activated by E1a share no common sequence homology. This lack of a E1a recognition site in the promoters led to the view that E1a activated transcription by a number of indirect means, i.e. either through an increase in the amount of cellular factors such as ATF and MLTF, which are known to have a role in transactivation, during adenovirus infection (Flint and Shenk, 1989). Alternatively, E1a may activate existing cellular transcription factors, an example being TFIIIC which is activated by phosphorylation in adenovirus infected cells. Support for this theory came from studies which showed that infection of cells with an adenovirus mutant unable to express the 289R protein did not result in phosphorylation of TFIIIC (Hoeffler et al., 1988). However,

such a mutant would have also been unable to synthesise other early proteins, so the lack of activation of TFIIIC may have represented an indirect consequence of the E1a defective phenotype.

Recent evidence has also pointed to a direct role for E1a transactivation, as part of a transcriptional initiation complex. Evidence from Ptashne et al (1988) revealed that a number of cellular transcription factors were composed of two functionally distinct domains, an N-terminal DNA binding domain and a C-terminal transcriptional activation domain, which can bind to other components of both the transcription and replication machinery. This concept was applied to E1a by Lillie and Green , (1989) who found that a hybrid protein, comprising the Gal4 DNA-binding domain and the acidic transcription activating domain of E1a, transactivated transcription from E4 and E1b promoters with high efficiency when Gal4 binding sites were introduced into the promoters of the adenovirus genome. However, since E1a doesn't bind to any nucleotide element in these promoters, it would seem that E1a influences the level of transcription by binding to a protein already bound at the promoter and not directly. This has been demonstrated in the case of the cellular transcription factor E2F (important for activation of genes required for and involved in S-phase functions), which undergoes a dramatic change in activity during adenovirus infection, mediated by E1a (Kovesdi et al., 1986). During this process, the 19KDa E4 gene product interacts with E2F enabling it to bind to the E2 promoter region so that transcription can proceed (Huang and Hearing, 1989 ; Bagchi et al., 1990 ; Raychaudhuri et al., 1990). In uninfected cells this DNA binding activity would normally be blocked by the retinoblastoma gene product (see below) which interacts directly with E2F (Bagchi et al., 1991 ; Bandara et al., 1991), resulting in the inhibition of E2F's transcription activity. E1a when present disrupts this interaction thereby freeing E2F to

bind to the E2 promoter along with the 19KDa E4 gene product. This results in a marked increase in the level of transcription.

E1a is also important in cellular transformation by adenoviruses. This process seems to occur as a sequential process, involving both E1a and E1b gene products, although E1a alone has the property of immortalising primary cells in culture (Van den Elsen et al., 1983). However, stable transformation of cells leading to tumour induction requires the presence of E1b, whose function can be substituted by other oncogenes including activating H-ras and the polyoma middle T-antigen. In addition E1a can be replaced by polyoma large T-antigen and the myc and p53 oncogenes (Ruley, 1983 ; Jenkins et al., 1984). Mutational analysis studies on E1a have shown that its transformation and transactivation activities are situated on different domains. The transformation properties have been mapped to two regions on E1a named CR1 an CR2 (Schneider et al, 1987 ; Fahnestock and Lewis, 1989), which are common to both the 289R and 243R proteins. Several other groups have also shown E1a to be associated with various host cell proteins referred to as anti-oncogenes or tumour suppressors. Prominent amongst these proteins is p105-RB, a product of the retinoblastoma gene (Harlow et al, 1986 ; Whyte et al, 1988a, 1989). The RB protein is thought to be a tumour suppressor protein as its corresponding loss of activity has been found in malignant cell types (Weichselbaum et al, 1988). Interestingly, the regions of E1a involved in p105-RB binding were mapped and correspond to those essential for cellular transformation (Whyte et al, 1988b, 1989). The oscillation of RB protein phosphorylation with the cell division cycle (Buchkovich et al., 1989 ; DeCaprio et al., 1992), binding of RB protein to oncoproteins like E1a, and the association of RB protein with potentially important cell cycle regulatory proteins may effect cell growth by affecting the cell division

cycle.The RB protein is thought to play some role at a restriction point in the G1 phase of cell division, either to allow exit from the cell cycle or delay entry into S phase until a suitable growth status is achieved (reviewed in Goodrich and Lee, 1993). In conclusion, it therefore appears that an important part of E1a mediated transformation occurs by the direct interaction of E1a with proteins involved in the regulation of cell growth and division.

The next mRNA's to be transcribed are the E1b, E2, E3 and E4 regions. These mRNA's are detected 1.5-2 p.i. and reach a maximum for E1b between 6-7 hours post infection and 3-4 hours post infection for E3 and E4. E2 mRNA transcription reaches a maximum between 3-4 hours post infection. E1b which is located between m.u. 4.6-11.2 is transcribed from the r-strand and is activated by E1a. Four transcripts are produced; 13s, 14s, 14.5s and 22s. All these mRNA's have common 5' and 3' termini (Virtanen and Petterson, 1985) and are produced by differential splicing of a single precursor. The 14s and 14.5s mRNA's are identical to the 13s species with the addition of a third exon. The 22s mRNA contains two AUG's which are used during translation to encode two proteins with molecular weights of 55KDa and 19KDa, with the latter encoded for by the 13s, 14s and 14.5s mRNA's (Bos et al, 1981). Additionally, all four mRNA's also contain the coding regions for IX, the 14.3KDa structural protein associated with the GON's hexons. However, the coding information for this protein is translated from an additional mRNA transcribed at intermediate (not early) times during infection Esche et al., 1980). The precise functions of the E1b proteins are still unclear. It has been demonstrated that the 55KDa protein can bind to a 34KDa protein encoded by the E4 region, forming a 55-34KDa complex which has a role in the transport of late mRNA's from the nucleus to the cytoplasm (Pilder et al., 1986 ; Bridge and Ketner, 1990). As with E1a, the protein products

from E1b are required for complete cellular transformation by adenoviruses (Van der Eb., 1984). Both the 19KDa and the 55KDa proteins are involved in transformation. The 55KDa protein binds to p53, a cellular anti-oncogene protein found at elevated levels in transformed cells (Sarnow et al., 1982), like E1a, can co-operate with activated ras genes in the malignant transformation of primary cells (Eliyahu et al., 1984 ; Parada et al., 1984). The E1b 55KDa protein is important for adenovirus transformation activity, as mutants in the 55KDa E1b gene are severely reduced in their ability to transform rodent cells (reviewed in Shenk, 1989). Interestingly, the region of p53 which binds to E1b 55KDa protein resides in the amino terminal acidic domain, which is associated with the transcriptional transactivating function of p53 (Fields and Jang, 1990 ; Raycroft et al., 1990 ; Unger et al., 1992). In fact, it has recently been demonstrated by Yew and Berk (1992) that the apparent binding of wild-type adenovirus type 2 E1b 55KDa and the equivalent adenovirus type 12 E1b 54KDa proteins to wild-type p53 severely inhibits the ability of p53 to mediate transcriptional transactivation, at the same time promoting transformation of rodent fibroblasts in conjunction with E1a. Mutants of E1b 55KDa and 54KDa which do not bind p53 allow p53 to transactivate and are severely reduced in transformation potential in cooperation with E1a. The prevention of the normal tumour suppressor activity of p53 by E1b binding should result in a more actively dividing cell. Recently, Yew et al., (1993), using a fusion of E1b 55KDa and the Gal4 DNA-binding, have shown that E1a may prevent p53 activity by acting as a direct transcriptional repressor that is targeted to p53 responsive genes by binding to p53.

Additional studies on cellular transformation, with a large number of point and deletion mutants of the p19 gene, resulted in a range of mutant phenotypes which included degradation of all host cell and viral DNA,

abnormal cytopathic effect and formation of large plaques (White et al., 1988). Transfection studies using p19 expressing vectors indicated that the encoded 19KDa protein, (located within the nuclear, endoplasmic and plasma membranes, was associated with and profoundly disrupted the vimentin-containing intermediate filament network and the nuclear lamina (White and Capriani, 1989, 1990). It has been proposed that anchorage independent growth of adenovirus transformed cells results from such altered structural organisation of intermediate filaments induced by the 19KDa protein. Additionally, modification of the nuclear lamina by the 19KDa protein could be important in maintaining the integrity of both cellular and viral DNA after infection as E1b defective virus was found to contain degraded DNA.

The E2 transcription region, located between map units 75.7-11.3, is transcribed from the l-strand (Chow et al., 1979a, 1979b). During adenovirus infection E2 mRNA's are transcribed at early times from a promoter at map unit 75 which is dependent on E1a, then at later times from a promoter at map position 72 which is E1a independent.The E2 transcripts are divided into two sets depending on the position at which they terminate. The transcripts terminate at two different poly-adenylation (polA) sites and are termed E2a and E2b. E2a mRNA's are transcribed early in infection from a promoter at m.u. 75.4 and from a second promoter at 72.2, later in infection. Both terminate at a polA site at 62.4 (Baker et al., 1979). E2b mRNA's bypass this poly-A site and terminate at 11.3 (Stillman et al., 1981). All the E2 mRNA's share a common leader at m.u.68.8.

Only one protein is encoded by the E2a mRNA's, the 72KDa viral single stranded DNA binding protein (DBP). DBP is a multifunctional phosphoprotein and plays important roles in DNA replication, the control of early and late transcription and viral host range. DBP's role in DNA

replication will be addressed in later sections. The role of DBP in early and late transcription is probably due to its ability not only to bind DNA (Van der Vliet and Sussenbach, 1975) and RNA (Cleghorn and Klessig, 1990), but on its ability to regulate its own production (Nicholas et al., 1982) as well as the transcription of the E1 and E4 region (Babich and Nevins, 1981). Limited proteolytic studies have demonstrated that DBP is preferentially degraded into a C-terminal domain and a highly phosphorlyated N-terminal domain (Schecther et al., 1980). The C-terminal region binds to DNA and is fully functional in DNA replication assays in vitro (Tsernoglou et al., 1985). Mutational studies on the N-terminal region of DBP indicate that this domain is not important for function in DNA replication, but in viral host range. Early studies by Klessig and Grodzicker, (1979) showed that human adenoviruses, which normally undergo an abortive infection in monkey cells, could replicate normally in these cells only when mutant N-terminal DBP was present. This was later found to be due to the presence of a functional fibre protein, not present in monkey cells infected with wild type virus (Anderson and Klessig, 1984).

Three mRNA's are transcribed from the E2b region. Two of these mRNA's encode the 80KDa precursor terminal protein (pTP) and the 140KDa DNA polymerase (Ad.Pol), which are present in infected cells as a stoichiometric complex and are required for DNA replication. The third mRNA encodes a 22.8KDa protein, whose function has not been identified as yet.

The E3 transcription unit maps from m.u. 76.8-85.9 on the r-strand (Berk and Sharp., 1978). Nine mRNA's are transcribed from this region through differential splicing of two precursor molecules. The E3 proteins produced are thought to be involved in the evasion of host cell antiviral defences, as well as the down regulation of epidermal growth factor

receptor (EGF-R). So far only three proteins have been assigned to the E3 region:- a 19KDa glycoprotein (Andersson et al., 1985 ; Burget et al., 1987 ; Rawle et al., 1989) which has been shown to bind to class 1 major histocompatability complex antigen, preventing their transport to the cell surface; a 14.7KDa protein which prevents cytolysis by the tumour necrosis factor (TNF) in infected cells (Gooding et al., 1988), and a 11KDa protein which down regulates the EGF-R, resulting in a subsequent decrease in the cellular metabolism of infected cells (Carlin et al., 1989 ; Tollefson et al., 1990).

mRNA transcripts from the E4 region are transcribed from the l-strand between m.u. 99-91.3 (Berk and Sharp, 1978). A primary transcript is produced which is then spliced into 12 mRNA's all with identical 5' and 3' ends (Virtanen et al., 1984). Generation of mutants containing large deletions resulted in pleiotropic including defects in late protein synthesis, in late mRNA accumulation and the shutdown of host cell protein synthesis (Halbert et al., 1985). Data from DNA sequencing analysis on E4 mRNA's has revealed that the existence of seven open reading frames (ORF's) encoding proteins ranging from 6-34KDa in size (Fryer et al., 1984 ; Virtanen et al., 1984).

E4 transcription is activated by a product of the E1a region (Jones and Shenk, 1979) and inhibited by the 72KDa DBP E1b product (Handa et al., 1983). Further studies have revealed that the 14KDa and 34KDa E4 products are involved in the accumulation of late viral mRNA's and in the shutdown of host cell macromolecular synthesis (Huang and Hearing, 1989). As mentioned above , the 34KDa protein has also been found to be associated with the 55KDa protein encoded by E1b in infected cells (Sarnow et al., 1984) and is involved in the transport of late mRNA's from the nucleus to the cytoplasm (Pilder et al., 1986). Another E4 protein with a molecular weight of 19KDa can be found in complex with

the cellular transcription factor E2F, with which it transactivates the level of transcription from the E2a promoter by increasing the DNA binding affinity of E2F for its recognition site (Marton et al., 1990).

3.2. Intermediate and late gene expression.

The E2b region, which codes for the viral proteins pTP and Ad.DNA Pol, is transcribed at intermediate times during infection from its own promoter between m.u. 23-30 and is terminated when mRNA production has finished (Lewis and Mathews, 1980). Two additional intermediate mRNA's are transcribed giving rise to two proteins; IX and IVa2, a 50KDa protein involved in virion morphogenesis. Although these mRNA's can be detected prior to DNA replication, maximal transcription occurs only after DNA replication (Crossland et al., 1983).

After the onset of DNA replication, transcription from early sites is increased 3-10 fold presumably due to an increase in the number of DNA templates (Shaw and Ziff, 1980). DNA replication also brings about a thousand fold increase in the activity of the major late promoter (MLP), from which the majority of mRNA's are transcribed. The MLP is situated on the r-strand at m.u. 16.5 (Ziff and Evans, 1978). The 5' ends of the mRNA's produced by the MLP consists of a 210b.p. tripartite leader sequence, which is spliced at numerous sites, to generate a large number of unique mRNA's. The mRNA's transcribed from the MLP are classified into five families named L1 to L5, with each family sharing a common poly-A site (Evans et al., 1977). The primary transcript for the families extends to m.u. 99, although the last poly-A signal (for L5) is at 91.3 (Fraser et al., 1979).

L1 transcripts are expressed at early times in infection generating 3 mRNA's which share a poly-A site at m.u. 39.3 (Fraser et al, 1982) and

5' cap sites at positions 29, 31 and 34. The transcripts also contain an additional leader sequence, containing an open reading frame between positions 21.5-23. The L1 mRNA's encode two structurally related proteins with molecular weights of 52KDa and 55KDa whose function is unclear (Miller et al., 1980). The third mRNA codes for IIIa, a 66KDa protein found associated with the hexon in mature virions (Akusjarvi and Pettersson, 1981) .

The L2 region generates 3 mRNA's which are transcribed from map position 39, have 5' cap sites at 39.4, 44.1, 45.9 and a poly-A signal at at m.u. 50 (Alestrom et al., 1984). The mRNA's encode for the important structural proteins: III (85KDa), VII (18.5KDa) and V (48.5KDa), (Miller et al., 1980 ; Akusjarvi and Petterson, 1981). All are structural components of the mature virion, III forming the penton base and VII and V constituting the two major proteins of the viral core. L2 also contains a fourth open reading frame (ORF) which codes for a hypothetical polypeptide 85 amino acids long which is thought to be the precursor for protein Mu, a component of the viral core.

Region L3 transcribes 3 mRNA's with 5' cap sites at 50.1, 52.3 and 60.2 with a poly-A signal at 62.4. The mRNA's encode for a single precursor which gives rise to a precursor of VI, hexon (II) and a 23KDa viral endopeptidase which specifically cleaves a number of precursor proteins to generate mature viral proteins: VI, VII, VIII and the terminal protein (TP). Recent inhibitor profile studies have shown that the 23KDa protein is a cysteine protease, which cleaves the target amino acid sequence (M,L,I)XGX-G (Webster et al., 1989 ; Webster et al., 1993).

L4 transcribes four mRNA's which have a common poly-A signal at 78.5. Only 3 proteins have been assigned to three of the mRNA's: the mRNA's encode; a 100KDa multifunctional protein involved in hexon assembly and inhibition of host messenger translation (Gambke and

Deppert, 1984 ; Adam and Dreyfus, 1987), a 33KDa non-structural protein whose function is unknown and a 26KDa precursor of VPVIII, a structural protein associated with the hexon (Miller et al., 1980).

L5 contains two major mRNA's transcribed between m.u. 86-91.3 (Miller et al., 1980). These mRNA's differ from the other late transcripts by virtue of the fact that they contain leader sequences derived from map positions 72.2, 78.6 and 84.7, in addition to the tripartite leader sequence. These mRNA's produced encode the IV fibre protein (Uhlen et al., 1982).

Two additional low molecular weight mRNA's are transcribed from the adenovirus genome late in infection and are designated virus associated RNA's I and II (VA RNA I AND II). These RNA's are transcribed on the r-strand from two promoters at map positions 29.5 and 30.2, which are located within the non-coding region of L1. They are transcribed by RNA polymerase III and can form complex secondary stem-loop structures via base-pairing. Mutational studies on both the VA I and II promoters, demonstrated that the VA RNA II species was non-essential, whereas VA RNA I was essential, for the efficient translation of late viral transcripts (Thimmapaya et al., 1982). It seems that VA RNA I acts to facilitate late translation by maintaining the activity of the eukaryotic translational initiation factor eIF-2, which is inactivated when phosphorylated by a double stranded RNA activated kinase (DAI), (Akusjarvi et al., 1987). It is believed that VA RNA I achieves this by preventing the activation of the kinase, thus allowing translation to continue (O'Malley et al., 1989). Further studies on mutant VA RNA I molecules have revealed that it consists of two domains which have different functions, one mediates binding to DAI and the other directly interferes with DAI inactivation (Mellitis et al., 1990).

4. Adenovirus DNA replication.

The initial impetus for studying adenovirus DNA replication in depth arose from the idea that analysis on the mechanism of replication of the small genomes of eukaryotic infecting DNA viruses could provide insights into the mechanisms involved in the replication of larger and more complex host cell genomes. To date adenovirus DNA replication has been extensively studied both in vivo and in vitro.

Adenovirus DNA replication can be detected 6-8 hours post infection, usually reaching a maximum after 19 hours. Replication takes place in the host cell nucleus and is found to be coincident with a complete shutdown (90%) of host cell DNA synthesis. After 24 hours, approximately 10^5-10^6 new double stranded DNA daughter molecules are present, equalling the total DNA content of the host cell (Green et al., 1970). Ultimately, only 20% of the progeny genomes are packaged into mature virions. A lot of the important mechanistic features of adenovirus DNA replication were elucidated by studies on viral DNA replication in vivo. However the development of in vitro assays capable of replicating DNA in vitro (Challberg and Kelly, 1979) has allowed the identification, purification and characterisation of three viral gene products and four host cell polypeptides that have been found to be necessary for the synthesis of the adenovirus genome.The in vitro systems have also led to the elucidation of important nucleotide sequences within the adenovirus genome important for DNA replication. The following sections will deal with the evolution of both the in vivo and in vitro Ad. DNA replication systems.

4.1. Adenovirus DNA replication in vivo.

Most of the initial studies utilised both pulse-labelling and electron microscopic techniques to study the mechanics of adenovirus DNA replication in infected cells.

Initially, pulse-labelling studies were used and identified replicative intermediates in adenovirus infected cells which had been exposed to [^{3}H] thymidine. When labelled DNA from these cells was examined by native sucrose gradient centrifugation, it was observed that a significant proportion of the DNA had a greater sedimentation rate than mature viral DNA (Pearson and Hanawalt, 1971). These premature viral DNA molecules also had a greater buoyant density in caesium chloride gradients suggesting the presence of single-stranded regions in the replicative intermediates (Pearson and Hanawalt, 1971; Robin et al., 1973). This was confirmed when S1 nuclease treatment abolished the difference in density (Pearson, 1975). When the same experiments were carried out under denaturing conditions, a wide range of DNA strands of various sizes were present in the intermediates (Pearson and Hanawalt, 1971).

Early electron microscopic analysis by various groups (Lechner and Kelly, 1977) revealed the presence of two classes of replicating molecules; Type I intermediates containing genomic length DNA, with between one and four single-stranded branch structures, and Type II intermediates which consist of linear molecules with a single-stranded region extending from one end. Early studies by Ellens et al., (1974) demonstrated that when adenovirus replication was synchronised after the release of a hydroxyurea block, branched (Type I) intermediates predominated early and unbranched molecules at later times. Investigations on the level of initiation of DNA replication in these

intermediate molecules by partial denaturation techniques (Lechner and Kelly, 1977 ; Revet and Benichou, 1981) revealed that the DNA molecules initiated with equal frequency at either end of the genome. The polarity of DNA replication in the intermediates was then determined by Lechner and Kelly (1977) who demonstrated that terminal transferase could add labelled dTTP residues to the termini of growing DNA molecules, forming poly (dT) tails.These tails were observed growing only at the branch points of Type I molecules and at double to single-stranded transitions of Type II molecules, indicating that the daughter strands contained only one free 3'-OH group which is located at their growing points. The conclusion drawn from this was that both Type I and Type II DNA synthesis proceeded in a 5' to 3' direction.

A model for adenovirus DNA replication proposed by Lechner and Kelly, (1977) was improved on by the finding that the inverted terminal repeats (ITR 's) of the displaced strands in the replicative intermediates could associate, giving rise to a single-stranded circle of DNA with a double-stranded region, known as a "panhandle" structure (Daniell, 1976). Supportive evidence for panhandle formation came from transfection studies by Stow (1982a) and Hay et al., (1984). Stow (1982a) showed that Ad.2 genomes with deletions within the left ITR would generate progeny virus with two intact ITR's after transfection into permissive cells. This evidence was substantiated by the work of Hay et al., (1984) using a linearised plasmid containing inverted repeats of a 94bp E.coli DNA fragment with a 570bp Ad.2 terminal fragment at one end. After cotransfection of this plasmid into permissive cells with Ad.2 wildtype DNA, progeny molecules an extra 0.5kb in length were generated. This indicated that during adenovirus infection the ITR's at each end do interact. Incorporation of this transfection data into the Lechner and Kelly model suggested that the ITR's at either end of the

genome did indeed interact to form panhandle structures during infection. The bulk of in vivo data has indicated that adenovirus DNA replication initiated at the 5' end of each daughter strand, at an origin located at or near the termini of the parent DNA molecule and terminated at the opposite end of the genome with the concomitant displacement of the strand not being utilised as the template.

4.2. Adenovirus DNA replication in vitro.

The development of an adenovirus DNA replication system totally dependent on an exogenous template for initiation and elongation has made it possible to purify and develop functional assays for the individual components of the replication system. As well as allowing a precise breakdown of the major features of the replication pathway, the in vitro assays have confirmed many of the earlier models based on in vivo data.

Early in vitro studies were based on attempts to isolate Ad.2 replication complexes (replicating DNA and replication proteins) from infected nuclei. However these attempts were unsuccessful as the DNA isolated became fragmented during the isolation procedure (Frenkel, 1978). The complexes were prepared by various chemical treatments; e.g. Kaplan et al (1977) isolated a soluble extract containing replicating DNA by fractionation with ammonium sulphate. The isolated complexes required both Mg^{2+}, dNTP's and a large amount of the viral 72KDa DBP protein before they would synthesise DNA (Yamashita et al., 1977 ; Kaplan et al., 1977). However, a major drawback of these early assay systems was that the elongation of daughter DNA strands in vitro, could only proceed on molecules which had already been initiated in vivo. This fact combined with the short "half-life" of the complexes, after extraction,

made these early in vitro systems unsuitable for further analysis of the replication mechanism.

A major breakthrough in the study of adenovirus DNA replication was the development by Challberg and Kelly (1979) of an in vitro replication system consisting of cell-free soluble extracts from the nuclei of Hela cells isolated from adenovirus type 5, Ad.5 DNA-protein template in the presence of both ATP and Mg^{2+}. This represented the first working adenovirus DNA replication system in which both the initiation and elongation of DNA replication occurred entirely in vitro.

4.3. A model for the mechanism of adenovirus DNA replication : "Protein-priming mechanism".

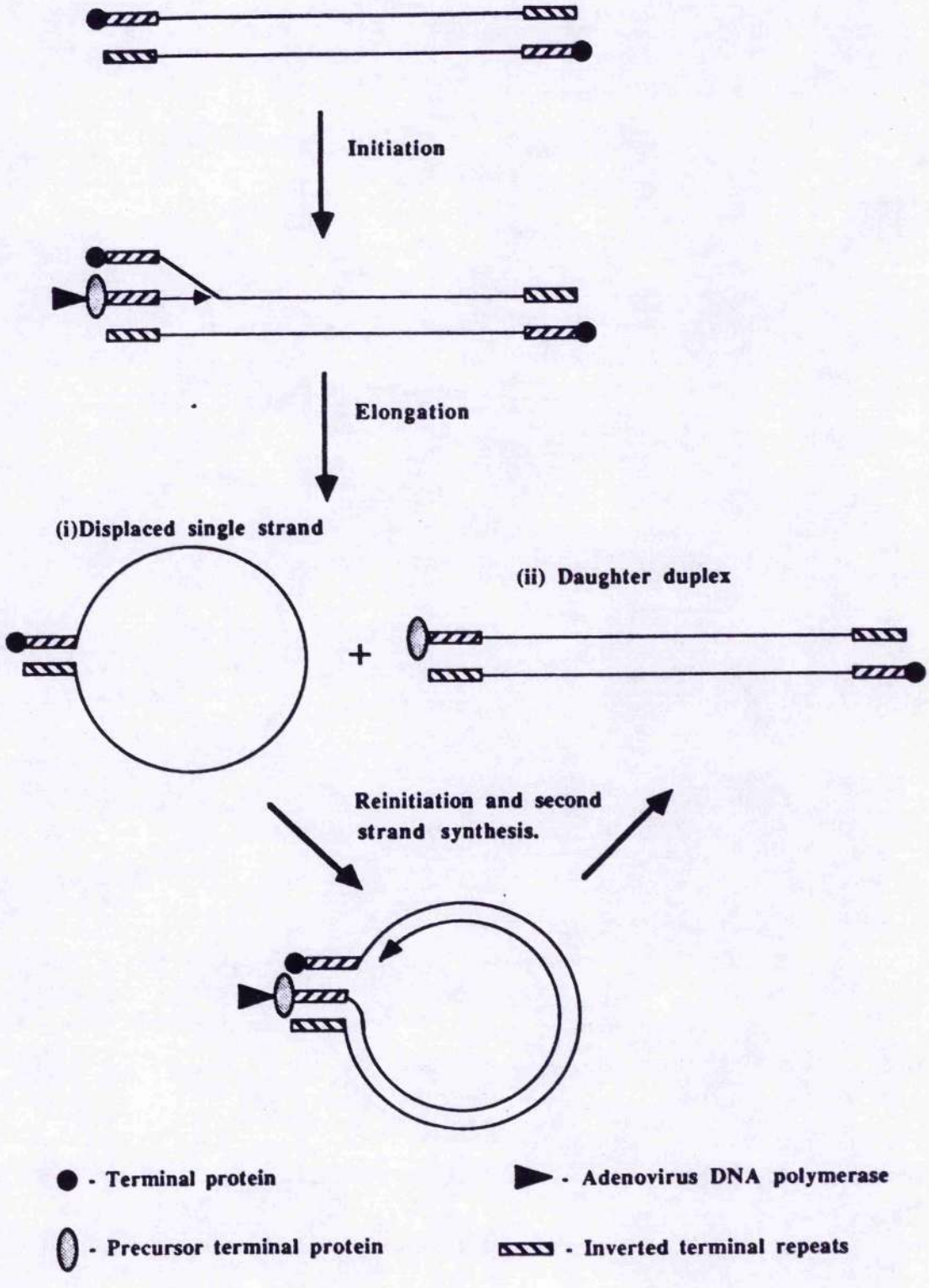
Replication of the linear 36Kbp adenovirus genome (reviewed: Hay and Russell, 1989 ; Stillman et al., 1989) initiates via a protein-priming model first proposed by Rekosh et al., (1977). Initiation starts at the origins, which are located at the ends of the genome, by the covalent linkage of dCMP to the β-hydroxyl group of a serine residue in the terminal protein (TP) or to be more exact, in its precursor (pTP), by the adenovirus DNA polymerase (Ad.DNA pol.). The polymerase then proceeds to synthesise a new strand starting from the 3'-OH end of the dCMP moiety (reviewed in Salas, 1991). The non-template strand is displaced during this reaction and the adenovirus DNA-binding protein (DBP) binds this displaced single-strand and protects it from nuclease digestion (Van der Vliet et al., 1977). The molecular ends of the totally displaced ssDNA may subsequently hybridise to each other via its long inverted terminal repeat (ITR) sequences (Hay et al., 1984). The resulting partially double-stranded DNA, called a "panhandle", contains a functional dsDNA origin sequence at its ends. Replication from this origin results in the formation of a fully duplex daughter molecule (Leegwater et al.,1988). However, it

is not clear whether panhandle formation is an absolute requirement for second strand synthesis since it has been demonstrated in vitro that initiation can also take place on single-stranded templates (Kenny and Hurwitz, 1988). It is thought that the complex of DBP with the displaced ssDNA strand forms a preferred substrate for the incoming DNA polymerase. Late during infection, the precursor terminal protein is cleaved by a 23KDa virally encoded protease to its 55KDa mature terminal protein form. A schematic diagram of this model is shown in figure 3.

5. Ad.2 / Ad.5 DNA replication origins.

Mutational analysis of plasmids containing the terminal sequences for Ad.2 and Ad.5, which possess nearly identical origin sequences, in DNA replication assays (in vitro) revealed the existence of three different functional domains within the terminal 51 base-pairs of the viral genome important for efficient DNA replication. The first domain consists of the first 18 base-pairs of the genome and constitutes the minimal replication origin. This "core" region is very A-T rich and contains the sequence 5'-ATAATATACC-3' (between nucleotides 9 and 18) which is fully conserved in different adenovirus serotypes (Tamanoi and Stillman, 1983 ; Challberg and Rawlins, 1984). Base-pair changes within this conserved sequence greatly reduced the template's ability to replicate. This conserved region between nucleotides 9 and 18 has been implicated in binding to the preterminal protein (Temperley et al., 1992). The second region comprises the region of DNA sequence between nucleotides 19-39 and is the binding site for the cellular transcription nuclear factor I (Nagata et al., 1983). The presence of this domain, which is present in some, but not all adenovirus serotypes (except Ad. 4, Ad.9 and Ad.11), increases the efficiency of initiation of replication. The third domain

Figure 3. A model for adenovirus DNA replication with schematic representations of viral proteins shown.
Lines with arrow heads represent newly synthesised DNA. The arrow indicates the direction of synthesis.


Initiation
Elongation
(i)Displaced single strand
(ii) Daughter duplex
+
Reinitiation and second strand synthesis.
● - Terminal protein
▶ - Adenovirus DNA polymerase
- Precursor terminal protein
- Inverted terminal repeats

found between nucleotides 39 and 51 is highly conserved and corresponds to the nuclear factor III binding site. It is not essential , but stimulates the initiation of DNA replication (Pruijin et al., 1986). The roles of NF-I and NF-III in DNA replication will be discussed more thoroughly in a later section. The origin requirements for Ad.2 have also been defined in vivo using a system in which transfected plasmids containing cloned origin sequences are amplified in the presence of a full complement of viral replication proteins provided by co-transfected helper virus (Hay, 1984). This technique was initially developed for the isolation of other eukaroytic origins of replication, such as SV40 (Myers and Tjian, 1980) and HSV (Stow, 1982b). A series of plasmids containing deletions in the Ad.2 ITR were used in such an assay, with Ad.2 helper virus, and revealed that DNA molecules containing ITR sequences deleted to within 45bp of the terminus were replicated as efficiently as those containing a complete ITR sequence. However, plasmids containing ITR sequences containing ITR sequences deleted to within 36bp of the terminus were not replicated, demonstrating that all the sequences required for an origin of Ad.2 DNA replication in vivo are contained within the terminal 45bp of the genome which contains the 1-18 "core" domain and the NF-I recognition site (Hay, 1985a, 1985b). In a further study in which ITR's containing such deletions were reconstructed into viral genomes it was found that genomes containing deletions which removed the NF-I site were non-infectious whereas genomes containing the terminal 45bp were fully infectious (Hay and McDougall, 1986).

6. Viral proteins required for adenovirus DNA replication.

Three viral proteins are required for adenovirus DNA replication in vitro; the single-stranded DNA-binding protein (DBP), the adenovirus DNA polymerase (Ad.DNA pol) that forms a heterodimer complex with the

third viral protein, preterminal protein (pTP). All three of these proteins are transcribed from the E2 region on the adenovirus genome. There are two spliced mRNA transcripts produced from this region ; E2a, which encodes DBP and E2b which transcribes a 22kb mRNA capable of encoding for both the pTP and Ad.DNA pol. proteins. Both pTP and Ad.pol. are expressed at the same time during infection to help heterodimer formation which is needed for efficient DNA replication. The roles of each of the proteins in adenovirus DNA replication will be discussed in the following sections.

6.1. Terminal protein and its precursor: Protein priming of DNA synthesis.

Using electron microscopy Robinson et al., (1973) identified the presence of a protein linked to the 5' termini of mature adenovirus DNA. The 55KDa terminal protein (TP) identified was shown to be covalently linked to the viral DNA by virtue of the resistance of the DNA-protein complex to boiling and SDS treatment. However, early in vitro DNA replication studies indicated the presence of an 80KDa precursor terminal protein (pTP) covalently attached to the 5' end (Challberg et al., 1980). Analysis on an Ad.2 virus protease temperature sensitive mutant (H2ts1), which can replicate efficiently at its restrictive temperature, but whose progeny virions are not infectious, finally revealed that the 80KDa pTP was processed to the mature TP form late in virion assembly (Stillman et al., 1981). Furthermore, sequence analysis on the tryptic peptide maps of both virion purified pTP and TP indicated a great deal of amino acid homology suggesting that the 80KDa species was indeed the precursor of the 55KDa terminal protein. Recent studies on the maturation of precursor terminal protein have revealed that this processing event is catalysed by a virally encoded 23KDa cysteine protease (present in all the

adenovirus serotypes) that is activated by a virus coded disulphide-linked peptide (Webster et al., 1993). The involvement of precursor terminal protein (pTP) in the initiation of DNA replication was demonstrated by Lichy et al., (1981) when it was shown that a covalent complex between pTP and [^{32}P] labelled dCMP, the 5' terminal nucleotide of the nascent strand, was formed in a cell free extract from infected cells in a reaction which required viral DNA-protein complex as template, $MgCl_2$, ATP and dCTP. pTP capable of forming initiation complex was first purified from infected Hela extracts by DNA affinity chromatography (Enomoto et al., 1981).They found it in a tightly bound complex with a 140KDa protein from which it could only be dissociated by sucrose density centrifugation under denaturing conditions in the presence of 1.7M urea (Lichy et al., 1982). Closer investigation of the 140KDa protein confirmed that it was the adenovirus DNA polymerase (Field et al., 1984).

Further studies by Rijinders et al., (1983) using an antiserum raised against virion purified TP showed that it could inhibit both pTP-dCMP complex formation and the specific labelling of Ad. DNA-protein terminal fragments. The same anti-TP serum was shown to be ineffective against the polymerase activity of purified pTP-Pol complex. Van Bergen et al., (1983) then demonstrated using the same anti-TP serum that replication on a linearised plasmid containing the Ad.2 ITR's could be inhibited, indicating that inhibition was due to an effect on pTP and not the mature TP attached to the template. Although the role of pTP in the protein priming of Ad.2 DNA has been elucidated, the role played by the mature TP protein species in DNA replication is still unclear.

A role for pTP in the nuclear transport of adenovirus DNA polymerase has recently been found.Transfection studies by Zhao et al., (1988) revealed that pTP could facilitate the transport of wild type Ad.DNA polymerase into the nucleus through a direct interaction with

pTP. Even mutant Ad.DNA polymerase without a nuclear localisation signal was transported into the nucleus when pTP was present. The formation of these protein-protein complexes in the cytoplasm most probably represents an important mechanism for the location of replication proteins to their required destinations in the cell.

Initial observations indicated that viral DNA infectivity was increased when TP was present (Sharp et al., 1976). This observation was reinforced when a number of in vitro DNA replication studies (Tamanoi and Stillman. 1982 ; Van Bergen et al., 1983 ; Guggenheimer et al., 1984) reported that TP free DNA was less efficient as a template for initiation and replication than TP containing DNA. Additionally, it was found that the initiation reaction didn't always occur at the termini in protein free templates, but at any G residues between nucleotide bases 4 and 8 in the ITR. These experimental observations suggested several roles for mature TP in adenovirus DNA replication: 1. TP could have a role in protecting progeny viral linear DNA from digestion by exonucleases; 2. Interaction with the preterminal protein(pTP), which is thought to play a part in the correct positioning of the replication proteins at the origin of replication (Pronk and van der Vliet, 1993); and 3. Strand unwinding of the DNA helix template which was confirmed by Kenny et al., (1988). Recent evidence suggests that only a small fragment of the TP is required for the enhancement of DNA replication in vitro. Pronk et al., (1992) demonstrated by employing α-chymotrypsin digestion that a considerable part of the TP N-terminus could be lost before the enhancement effect was compromised.

Recent evidence suggests that both TP and pTP can mediate the binding of adenovirus DNA to the nuclear matrix. Initial in vivo studies by Schack et al., (1990) demonstrated that unlike wild-type TP-DNA viral DNA with a mutant TP attached was defective in nuclear matrix

binding and subsequently not efficiently packaged into mature virions. This hypothesis gained support when in vivo and in vitro studies by Fredman and Engler, (1993) revealed that vaccina virus purified pTP could compete with both nuclear matrix bound pTP and TP-DNA. This has suggested that there are at least two ways in which the matrix might aid in DNA replication. One possibility is that the nuclear matrix plays a structural role, providing a locale at which both the replication proteins and viral DNA can interact. Alternatively, the matrix may play a role by providing access to required factors (enzymes) embedded within its structure. However, the role of the matrix is probably structural, since the in vitro DNA replication system of adenovirus was developed in the absence of proteins from the nuclear matrix.

6.2. Adenovirus DNA polymerase.

During the purification of precursor terminal protein from an Ad.2 infected cytosolic extract (Enomoto et al., 1981) two protein species which were essential for DNA replication were isolated. These were; an 80KDa species found to be preterminal protein and a 140KDa protein with a unique DNA polymerase activity distinguishable from various cellular DNA polymerase species (α, β and γ). These two proteins copurified in a tight heterodimer complex which only separated into its component parts after fractionation on a glycerol gradient containing 1.7M urea. This was confirmed by Lichy et al., (1982) who also demonstrated that the DNA polymerase activity resided in the 140KDa species and didn't require the 80KDa pTP protein species.

The viral origin of the DNA polymerase was first suggested from complementation experiments with a group of temperature sensitive E2 transcription mutants of Ad.5 (Group N mutants) defective in both the initiation and elongation of DNA replication in vivo (Stillman et al., 1982

; Van Bergen et al., 1983). Furthermore, nuclear extracts prepared from cells which were subsequently infected with these mutant viruses were unable to support pTP-dCMP complex formation in vitro. However, upon addition of the 140KDa DNA polymerase, replicative activity was restored to the extracts (Stillman et al., 1982).

Once purified to homogeneity the DNA polymerase underwent rigorous biochemical characterisation. Comprehensive studies carried out by Field et al., (1984) revealed that both the pTP-dCMP and DNA polymerase activities were inactivated in the presence of N-ethylmaleimide (5mM) or by heating at 55°C for 15 minutes. These early studies also seemed to indicate that the virally encoded polymerase resembled the eukaryotic DNA polymerase α as it used activated DNA (nicked) as a template and was sensitive to both cytosine β-D-arabinoside 5'-triphosphate and sodium chloride. However, it differed from DNA polymerase α when it was shown to be less sensitive to ddTTP and Aphidicolin. Further in vitro adenovirus DNA replication studies by Lichy et al., (1982) then revealed that both pTP-dCMP formation and chain elongation to the 26th nucleotide were unaffected in the presence of aphidicolin. However, extensive chain elongation was found to be affected by aphidicolin and resulted in the accumulation of DNA chains approximately 10kb in length (Nagata et al., 1983). The existence of an aphidicolin resistant viral polymerase was later confirmed when the E2b transcription region (containing both the pTP and pol mRNAs) was cloned and expressed in Cos cells. A 140KDa aphidicolin resistant DNA polymerase activity identical to that of the putative Ad.DNA polymerase was detected in extracts of transfected cells (Shu et al., 1987).

Fields et al., (1984) initially observed that the Ad. DNA polymerase could utilise a variety of homopolymer template-primers. Ad. DNA pol. could replicate on: poly d (C): oligo d (G), poly d (C): oligo r (I), poly d

(T): oligo d (A), and poly d (T): oligo r (A), but not on poly d (G), poly d (I) or poly d (GC) templates.

The purified Ad.DNA pol. was also found to contain a 3'-5' exonuclease activity whose probable function is to increase the fidelity of replication by removing mismatched nucleotides (Reha-krantz and Bessman, 1977). The exonuclease function which is shared with other viral and prokaryotic DNA polymerases (Bernad et al., 1989) was found to be 10-fold more active on single-stranded than duplex DNA (Field et al., 1984).

The presence of DBP was found to have a profound effect on both the DNA polymerase and exonuclease activities of the Ad. polymerase (Field et al., 1984) DNA synthesis on poly (dT) : oligo (rA) was stimulated 10-100 fold by the presence of DBP and a further 3-10 fold suggesting that it increased the processitivity of the Ad. polymerase. However, this effect was found to be template specific as DNA synthesis on activated DNA and poly (dC) : oligo (dG) were not affected by DBP. Other single-stranded binding proteins (SSB's) e.g. E.coli SSB were unable to substitute for Ad. DBP. Alternatively, it was found that Ad.DBP did not substitute for HSSB in stimulating the activity of human cellular polymerase α. These results were consistent with a reported specific cooperative interaction between DBP and the Ad.DNA polymerase (Lindenbaum et al., 1986). DBP is thought to act in this complex by stabilising the interaction between Ad. DNA polymerase and the DNA template. Indirect evidence for the formation of a physical complex came from work by Lindenbaum et al., (1986) who observed an increase in the thermostability of Ad.DNA pol in the presence of an excess of DBP. Although physical evidence of functional complexes between SSB's and DNA polymerases have been found in a variety of prokaryotes such as T4 and T7 (Huberman and Kornberg, 1971; Reuben

and Gefter, 1973) and eukaryotes like human SSB (Kenny et al, 1989, 1990) it has not yet been possible to isolate an Ad.DNA polymerase -DBP complex.

In addition to its polymerase function Ad.pol also possesses an intrinsic 3'-5' exonuclease activity, common to many prokaryotic and eukaryotic DNA polymerases (Bernad et al., 1989). This exonuclease which is thought to have a role in the proof-reading of nascent DNA during elongation, was found to be inhibited up to 7-fold by the presence of DBP. The nature of inhibition by DBP was shown to be due to a direct effect upon the DNA polymerase rather than binding to and protecting the DNA (Lindenbaum et al., 1986).

Recent affinity chromatography studies by Bosher et al., (1990) have demonstrated that Ad.2 DNA polymerase can also interact with the cellular protein nuclear factor I (NF-I), which has been shown to stimulate the initiation of adenovirus DNA replication between 5-30 fold (Mul and Van der Vliet, 1990). Only Ad.pol and the pTP-pol heterodimer complex protein species were observed binding to a matrix containing NF-I immobilised on sepharose. pTP alone did not bind to the NF-I affinity column. These same conclusions were reached by Chen et al., (1990) who found that an antibody raised against Ad.DNA polymerase precipitated two protein species; Ad. DNA pol and NF-I. This protein-protein interaction is thought to play a role in the assembly of the preinitiation nucleoprotein complex at the adenovirus DNA origin of replication. The pTP-pol heterodimer can recognise the 1-18 adenovirus "core" origin sequence on its own and bind to it without the aid of NF-I, but at much lower levels (Temperley and Hay, 1992). It is thought to do this through the ability of the polymerase to bind DNA. Mutational analysis by Joung and Engler, (1992), has identified two putative cysteine-histidine rich clusters in the Ad.2 DNA polymerase, that affect

its DNA-binding and presumably polymerisation functions. N-terminal domain mutations in this enzyme had a moderate effect on both DNA synthesis and elongation, but failed to make the pTP-dCMP complex or bind DNA. C-terminal mutants had the greatest effect on both DNA synthesis, DNA-binding and pTP-dCMP complex formation.

Recent in vivo evidence by Ramadhandra et al., (1993) has shown that the Ad.DNA polymerase is a phosphoprotein with its activity modulated by both phosphorylation and dephosphorylation events. Using both in vivo phosphorylation and tryptic peptide map analysis, they demonstrated that Ad.DNA polymerase from adenovirus infected Hela cells and a vaccinia virus vector were phosphorylated on identical peptides at Ser 67. The importance of phosphate group(s) on its replication initiation activity (in vitro) was shown when dephosphorylation of the Ad.DNA pol by calf intestinal alkaline phosphatase prior to its addition to the reaction resulted in a significant decrease in pTP-dCMP complex formation. Additionally, they have demonstrated that the polymerase is phosphorylated by a stably associated Histone H1 kinase which exhibits properties similar to the cdc2 family of kinases (Ramachandra and Padmanabhan, 1993).

The adenovirus DNA polymerase shares regions of high amino acid sequence homology with a large number of DNA polymerases including several prokaryotic phage polymerases (bacteriophages T4 and ϕ 29), eukaryotic polymerases (human DNA polymerase α) and viral polymerases (adenovirus, herpes simplex and vaccinia virus). Four conserved amino acid regions designated I-IV (I being the most conserved between species) have been found in the adenovirus DNA polymerase. The most conserved regions (I-II) are found in the C-terminal domain of the protein and have been implicated in metal binding (region I) and substrate binding (regions II and III). However, it has to be

pointed out that the functional importance of these regions in the adenovirus DNA polymerase is only based on sequence homology (Bernad et al., 1987) and comparison with regions in mutant HSV DNA polymerases that confer altered sensitivity to nucleotide analogues such as aphidicolin and acyclovir (Larder et al., 1987 ; Marcy et al., 1990).

6.3. Adenovirus DNA binding protein (DBP).

The relatively high abundance of DBP during the adenovirus infection cycle (maximum of $5x10^6$) helped make it become the first of the DNA-binding replication proteins to be identified and subsequently purified. Van der Vliet and Levine, (1973) initially reported the isolation of two polypeptide species of 72KDa and 48KDa from cells infected with Ad.5. No DBP was found in uninfected cell extracts. Further investigations revealed that the smaller of the two species was the proteolytic breakdown product of the larger (Levinson and Levine, 1977).These initial studies indicated that the two protein species bound specifically to single-stranded DNA (ssDNA), but not duplex DNA (ds DNA). However, more in depth DNA binding studies revealed that only the 72KDa full-length DBP species bound to both ssDNA and dsDNA (Fowlkes et al., 1979 ; Schechter et al., 1980). DBP binding was also found to be specific for the termini of a linear DNA template, with closed circular DNA molecules not being bound to any great extent. However, EM studies by Kedinger et al., (1978) revealed that only the single-stranded regions of DNA in replicative intermediates isolated from infected nuclei were coated with DBP, suggesting that in vivo the binding of DBP to DNA differed from that observed in vitro. More recent in vitro binding studies indicate that DBP can also bind to RNA (Cleghorn and Klessig, 1986). Although DBP apparently has a molecular weight of 72KDa this was subsequently shown to be an aberrant electrophoretic

mobility, with its true molecular weight as predicated from amino acid composition being around 59KDa (Kruijer et al., 1981).

The essential functional role played by DBP in adenovirus DNA replication was directly demonstrated in early in vitro systems where addition of anti-DBP antiserum was observed to inhibit elongation of nascent strands in isolated nuclei (van der Vliet et al., 1977). Further in vitro support for this idea came from the use of two temperature sensitive mutants of Ad.5: H5ts125 and H5ts107. Nuclear extracts from both of these mutants were found to be defective for full-length Ad.DNA replication on both exogenous and endogenous templates (Friefeld et al., 1983). In both cases activity was restored by the addition of purified wild-type DBP.

Although the role of DBP in elongation has been well documented over the years by both in vivo and in vitro studies, its role in the initiation of adenovirus DNA replication is still unclear. Shortly after the development of an in vitro initiation system (Challberg and Kelly, 1979) it was demonstrated that the addition of DBP to crude infected cell extracts, had no effect on the formation of the pTP-dCMP complex indicative of successful initiation (Lichy et al., 1981). Friefeld et al., (1983) subsequently showed that extracts derived from temperature sensitive mutant H5ts125 infected cells could in fact support pTP-dCMP complex formation to wild-type levels at the non-permissive temperature. These same extracts were unable to elongate on in vivo initiated DNA templates (Friefeld et al., 1983 ; Van Bergen and Van der Vliet, 1983). This suggested that DBP was dispensable for initiation but not elongation. However, more recent studies have observed a stimulatory effect on the level of initiation when DBP is present (Kenny and Hurwitz, 1988 ; Cleat and Hay, 1989 ; Mul and van der Vliet, 1993). The most likely explanation for these reported differences is that DBP

acts on initiation in a concentration dependent manner. At saturating concentrations DBP is probably inhibiting initiation by saturating the DNA template, thus preventing protein-protein interactions between the other replication proteins, required for their correct positioning at the origin of replication. A stimulatory effect at subsaturating concentrations could be due to a functional interaction between DBP and NF-I, a cellular DNA-binding protein which has also been found to stimulate the initiation of DNA replication. Evidence for the latter came from De Vries et al., (1985) who observed that NF-I 's ability to stimulate initiation in vitro was influenced by the concentration of DBP. Subsequent experiments demonstrated that the effect was cooperative in nature, suggesting that there was some kind of specific protein-protein interaction between the two proteins (Cleat and Hay, 1989 ; Stuiver and van der Vliet, 1990). However, no direct evidence for this interaction was found, either in vivo or in vitro. An alternative theory by Cleat and Hay, (1989) explained the effect as a result of DBP's ability to alter the structure of the DNA it bound to, i.e. DBP binds and alters the structure of the DNA in a specific way that facilitates NF-I binding. Support for the latter has come from recent in vitro structural studies on the nature of d.s.DNA-DBP complexes. Using a combination of electron microscopy, hydroxyl footprinting and circular dichroism Stuiver et al., (1992) have demonstrated that DBP can remove the tertiary structure of d.s. DNA fragments upon binding. This led to the suggestion that the structure of the DBP-DNA complex could well alter the hydrogen bond positions in the major and minor grooves, causing a fine tuning of the contacts between NF-I and the nucleotide bases, leading to a change in the NF-I binding affinity.

Recent studies by Mul and Van der Vliet, (1993) have reinvestigated the effect of DBP on DNA replication with respect to the

activities of both NF-I and Ad.DNA pol. They have proposed that DBP stimulates the initiation of Ad.DNA replication in two ways: by increasing the Vmax of the reaction at low NF-I levels, helping NF-I to bind DNA more easily, decreasing the Km of the polymerase for the initiator nucleotide dCTP. Km reduction could occur by DBP facilitating the unwinding of the replication origin, improving the presentation of important initiator nucleotides to Ad.DNA pol. or alternatively, DBP could induce a "better fit" for the initiator dCTP nucleotide in Ad.DNA pol. via either structural changes in the DNA or through a direct protein-protein interaction with the Ad.DNA polymerase. Indirect support for the former has come from earlier studies on Ad.DNA polymerase's sensitivity to the nucleotide analogue (s)-HPMPAapp (Mul et al., 1989). In these studies the sensitivity of Ad.DNA pol. for the drug was increased in the presence of DBP.

Partial chymotryptic digestion of purified DBP yields a C-terminal fragment of around 40KDa and a highly phosphorylated 27KDa N-terminal fragment (Klein et al, 1979) which are apparently functionally separate. Nucleotide sequence analysis on the C-terminal portion has revealed that it is conserved between all adenoviruses serotypes (Kitchingman et al., 1985 ; Vos et al., 1988). More detailed analysis on the C-terminal domain using temperature sensitive mutants of DBP revealed the presence of highly conserved "clusters" of amino acids which appear to be essential for DBP's functions in DNA replication. This supported earlier evidence by Ariga et al., (1980) and Friefeld et al., (1983), who both demonstrated that the C-terminal fragment alone was capable of binding DNA and stimulating DNA replication. Armed with this information, Neale and Kitchingman, (1990) created a series of mutants located in each of the three highly conserved regions of the C-terminal domain. These were all found to alter DBP's ability to bind to

single-stranded DNA templates in vitro. Further investigation revealed the presence of a zinc binding motif within this domain (between amino acids 273 and 286) which when mutated destroyed all of DBP's functions (Eagle and Klessig, 1992). More recent studies using limited proteolysis and photo-crosslinking techniques have revealed that the two residues, Methionine 299 and Phenylalanine 418, also play an important part in DBP's ability to bind ssDNA (Cleghorn and Klessig, 1992). The recent elucidation of the x-ray crystal structure of the C-terminal region (Tucker et al., 1994 Personal communication) should help in the further characterisation of DBP's role in DNA replication.

Mutations in the N-terminal domain do not seem to have the same effect on DBP's functions. Viruses carrying mutations in their N-terminal region have been shown to bind and replicate their DNA normally. However, the mutant viruses did show a change in their host range which was demonstrated as being due to the altered processing of the mRNA transcript encoding the fibre protein (Anderson and Klessig, 1984).

Sequence analysis on DBP from various serotypes has been completed and reveals that all appear to possess the characteristic two domain structure, with the N-terminal region showing the most variation between serotypes (30-40% at the amino acid level) and the C-terminal region being the most conserved (60-80%).

7. Cellular proteins involved in adenovirus DNA replication.

Limited initiation of DNA replication in vitro can occur in the presence of only the virally encoded pTP, Pol and DBP proteins (Rawlins et al., 1984). However, the addition of a crude nuclear extract of uninfected Hela cells was observed to restore efficient levels of initiation. This stimulatory effect was due to the presence of two cellular transcription factors, Nuclear factor I (NF-I) and Nuclear factor III (NF-III).

Transfection experiments indicate that NF-I and not NF-III are needed for in vivo DNA replication (Hay, 1985a ; Hay 1985b). Full elongation of the adenovirus DNA in vitro also requires the presence of the additional cellular factor, Nuclear factor II (NF-II).

7.1. Nuclear Factor I (NF-I).

Early in vitro DNA replication studies by both Ikeda et al., (1981) and Lichy et al., (1982) demonstrated the need for an unknown component in uninfected Hela cell nuclear extract, for optimal initiation and elongation in the presence of infected cytosol or purified pTP-pol heterodimer and DBP. An initiation and elongation enhancing activity was subsequently purified from the nuclear extract by Nagata et al., (1982). This 'active' fraction contained no detectable topoisomerase, nuclease, ATPase, RNA or DNA polymerase activities. The fraction contained a single major protein species with a molecular weight of 47KDa and was termed nuclear factor I (NF-I).

The early characterisation of NF-I concentrated on its ability to specifically bind the adenovirus origin. DNase I footprint analysis revealed that NF-I protected a region between nucleotides 17-48, which was subsequently found to be conserved between a number of adenovirus serotypes (Nagata et al., 1983 ; Rawlins et al., 1984 ; Leegwater et al., 1985). Extensive mutagenesis and deletion analysis on this NF-I binding site revealed that the protein actually bound to a smaller core consensus binding sequence 5'-TGGC (N_6) GCCAA-3' (Leegwater et al., 1985 ; Gronostajski et al., 1984, 1987 ; De Vries et al., 1985). Methylation and ethylation interference studies subsequently demonstrated that NF-I protected G and T residues in the major groove and on the phosphate backbone of the helix (De Vries et al., 1987). Further investigation revealed that the contacts made were similar in each of the two blocks of

symmetrical sequence and that inversion or replacement of the entire NF-I binding site had no effect on the efficiency of Ad.2 DNA replication in vivo or in vitro (Adhya et al, 1986 ; Hay et al, 1988). This suggested that NF-I bound to its site as a dimer, making base contacts on one side of the helix only (De Vries et al, 1987). Extensive sequence homology searches have shown that NF-I binding sites are also present in the promoter regions of eukaryotic genes such as the chicken lysozyme gene (Borgmeyer et al., 1984), human IgM gene (Hennighausen et al., 1985) and viral regulatory regions such as the CMV immediate-early promoter (Hennighausen and Fleckstein, 1986) and the MMTV LTR promoter (Nowock et al., 1985). In most of these cases deletion of the NF-I sites from these upstream regulatory sites resulted in a reduction in the level of gene transcription. Correspondingly, a five fold stimulation of transcription in vitro was observed when NF-I sites were inserted upstream of the TATA box of the adenovirus major late promoter (Gronostajski et al., 1988).

A wide range of proteins with NF-I like activity have been isolated from various sources. Analysis of extracts from human cells using affinity chromatography identified a number of NF-I like proteins ranging in size between 52-66KDa (Rosenfeld et al., 1987). Limited proteolysis revealed that these proteins had similar polypeptide patterns suggesting that they were all members of the same family. In addition, mRNA transcripts encoding NF-I like proteins have been found in rat, hamster and pig (Paonessa et al, 1988 ; Gil et al, 1988 ; Meisterernst et al., 1989). On the basis of immunological cross-reactivity data, amino acid composition and proteolytic cleavage patterns it appears that the NF-I proteins are indistinguishable from CCAAT-binding transcription factors (CTF's), a family of proteins involved in cellular gene transcription. Analysis of the cDNA's from various human NF-I / CTF

mRNA's have shown that they all originate from a single gene, giving rise to multiple mRNA transcripts by differential splicing of a precursor molecule (Santoro et al., 1988 ; Meisternst et al, 1989). The NF-I proteins had virtually indistinguishable DNA binding activities and stimulated Ad.2/Ad.5 initiation of DNA replication in vitro. Mutagenesis studies on the cDNA of the largest NF-I protein (CTF-1) identified two functional domains: a highly conserved N-terminal protease resistant domain 158 amino acids in length, which contains the functions for DNA-binding, dimerisation and DNA replication (Santoro et al., 1988 ; Mermod et al., 1989 ; Gounari et al., 1990 ; Bosher et al., 1991), and a less highly conserved, proline rich C-terminal domain which contains a transcriptional activation function (Mermod et al., 1989).

Binding of the N-terminal domain to DNA is sufficient to stimulate the initiation of adenovirus DNA replication (Bosher et al., 1991). However, activation of transcription requires the presence of both domains.The C-terminal region is presumed to be important for interaction either directly with components of the transcriptional machinery such as theTATA binding protein, (TBP), RNA polymerase or with transcriptional activator proteins, e.g. Sp1, AP-1. It has been demonstrated that transcriptional activation of a collagen type I promoter by transforming growth factor (TGFb) is mediated by an NF-I site, suggesting that it may be modified by an interaction with other transcription proteins into an active conformation allowing it to activate transcription (Rossi et al., 1988). This hypothesis has gained more recognition recently as NF-I binding sites have been found in the enhancer regions of Human Papillomavirus Type 16 (Apt et al., 1993), Human polyoma JC virus (Kumar et al., 1993) and Mouse mammary tumour virus (Mink et al., 1992). In each of these cases addition of NF-I led to transcriptional activation, but only in the presence of other activators that also had binding sites in the enhancer

regions. Although no direct physical evidence for protein-protein interactions between NF-I and these other activators exists it seems probable that these interactions play an important role in activation.

It is still not known if all the members of the NF-I/CTF family are exclusively involved with either DNA replication, transcription or both. However, it has been shown that an NF-I protein from chicken erythrocytes which can stimulate lysozyme transcription can also substitute for human NF-I in stimulating adenovirus DNA replication (Leegwater et al., 1986). This suggests that the NF-I/CTF proteins have a high degree of functional conservation amongst themselves, with their function in transcription and DNA replication being interchangeable.

The mechanism of stimulation of Ad. DNA replication by NF-I in vitro is complex (Mul et al., 1990). The degree of stimulation appears to be strongly dependent on the concentration of pTP-pol. At low pTP-pol concentrations, NF-I or NF-I-BD stimulated replication up to 50-fold, while at high concentrations stimulation was less than two-fold. This demonstrates that the in vitro need for NF-I can be overcome by high pTP-pol concentrations. This result provided an explanation for the different levels of stimulation by NF-I reported previously (Adhya et al., 1986 ; De Vries et al., 1985) as well as a strong indication for a direct interaction between NF-I and pTP-pol (see next paragraph).

The orientation and spacing between the NF-I site and the 1-18 core origin sequence is critical in adenovirus DNA replication. Insertion of additional sequence between these two sequence regions abolished NF-I mediated stimulation of DNA replication in vitro (Adhya et al., 1986 ; Wides et al., 1987) and in vivo (Bosher et al., 1990) . This suggested that a strict constraint on the spatial arrangement between the 1-18 core sequence and the NF-I site existed which was necessary to allow specific protein-protein interactions between NF-I and other

replication proteins. This was shown to be the case when both Bosher et al., (1990) and Chen et al., (1990) demonstrated that the Ad.DNA polymerase and NF-I made a specific protein-protein interaction on the adenovirus origin of replication. On the basis of their observations both concluded that the interaction between NF-I and Ad.DNA polymerase may serve to direct and stabilise the pTP-pol heterodimer complex into a preinitiation complex at the adenovirus origin of replication.

7.2. Nuclear Factor III (NF-III).

The host encoded cellular factor NF-III was initially identified in Hela nuclear extracts through its ability to stimulate the initiation of adenovirus DNA replication in vitro in the presence of NF-I (Pruijin et al., 1986). Purified NF-III has a molecular weight of 92KDa and when bound to its recognition site in the adenovirus origin stimulates the level of initiation in vitro 3-7 fold (O'Neill and Kelly, 1988). Deletion of this site resulted in a 30% decrease in the efficiency of replication in vitro. A combination of DNase I footprinting and methylation protection studies on Ad.2 identified the core binding site as, 5'-TATGATAA-3' which is situated between nucleotides 39 to 47 in the Ad.2 ITR. Mutational analysis has revealed that all of the bases within the recognition site are important as NF-III DNA-binding is reduced 2-10 fold when changed. This NF-III site which is partially conserved in human adenoviruses is not symmetrical and correspondingly it is believed that unlike NF-I, NF-III binds as a monomer (Pruijin et al., 1988). A direct relationship between NF-III DNA-binding and the level of initiation of DNA replication exists, i.e. Ad.2 contains a recognition sequence with a relative binding affinity 2.5 times lower than Ad.4 which contains the optimal binding site sequence 5'-TATGCAAAT-3'; correspondingly this leads to a lower level of stimulation of DNA replication.

NF-III also plays an active part in the regulation of transcription in many different systems. Sequence homology studies have shown it to be identical to members of the Octamer binding protein family a group of DNA binding proteins which recognise the consensus sequence 5'-TAATGARAT-3' (where R=A or G) and activate transcription. NF-III binds to its consensus sequence in a number of transcriptional regulatory gene elements e.g. the promoters of the IE genes of HSV, immunoglobulin ^{V}H and ^{V}L promoters and in the histone 2B promoter. In the case of the herpes simplex virus genes, a functional interaction between NF-III and a HSV viral component Vmw65 (VP16), along with a cellular protein complex forming factor (CFF), leads to transcriptional activation of the herpes virus (O'Hare and Goding, 1988 ; Preston et al., 1988 ; Arnosti et al., 1993).

NF-III's membership of the Octamer binding protein family was recognised by O'Neill et al., (1989) and Pruijin et al., (1989) who demonstrated that NF-III was functionally similar to another protein which bound transcriptional promoters and enhancers termed Octamer transcription factor (OTF-1). Pruijin's work demonstrated that both NF-III and OTF-1 had indistinguishable mobilities on SDS PAGE gels and could stimulate Ad.2 DNA replication. Also, antisera raised against OTF-1 could recognise NF-III and inhibit its stimulation of Ad.2 DNA replication. Many more Octamer binding proteins have been found and identified as having a common, conserved DNA binding region known as the POU domain. NF-III contains this domain, which like the N-terminal domain of NF-I is sufficient for the in vitro stimulation of Ad.2 DNA replication (Veriijer et al., 1990).Transcriptional activation by NF-III requires additional protein regions either through self-dimerisation or interaction with other POU containing proteins, such as Pit-1 (Verrijer et al., 1992). The actual POU domain can be subdivided into an N-terminal

74 amino acid POU-specific domain and a C-terminal POU homeodomain (Strum and Herr., 1988). It has been demonstrated that the POU homeodomain binds to DNA and inhibits DNA replication, suggesting that the POU specific domain is ultimately responsible for the stimulation of DNA replication.

NF-III's function in the initiation of adenovirus DNA replication is still not as defined as it is for NF-I. This has been based on observations which show that efficient initiation in vitro can exist in the absence of NF-III provided that NF-I is present (Rosenfeld et al., 1987). This was confirmed by studies which demonstrated that mutant Ad.2 viruses containing genomes with a deleted NF-III site were as infectious as wild-type virus with an intact NF-III site (Hay and McDougall, 1986). However, transfection assays on plasmids containing only an NF-III site , (no NF-I site) found that they could stimulate the basal level of Ad.2 DNA replication when compared to plasmids containing only the 1-18 core origin of replication (Hay et al., 1988). This confirmed in vitro replication studies by Mul et al., (1990) who found that both the NF-III and NF-I binding sites were needed for optimal stimulation of replication. Since the binding sites of the two proteins overlapped, this suggested that they could interact as a an NF-I / NF-III complex in a cooperative manner to stimulate DNA replication. However, since no evidence about any such an interaction exists it seems that both proteins can, by binding to their respective recognition sites, stimulate initiation independently and without steric hindrance (Mul et al., 1990). An alternative explanation that explains NF-III's ability to stimulate replication has been presented by Verrijer et al., (1991), who demonstrated that NF-III was able to bind the adenovirus DNA via its POU specific domain. This altered structure may enhance pTP-pol binding to the 1-18 "core" origin region of the genome. Alternatively, NF-III induced binding may position adjacently

bound proteins which the POU specific domain can interact with, such as VP16 (O'Hare and Goding., 1988 ; Preston et al., 1988) in such a way as to allow functional protein-protein interactions.

7.3. Nuclear Factor II (NF-II).

The search for another host encoded replication factor was carried out by Nagata et al., (1983) based on the observation that the products of DNA synthesis on terminal protein containing DNA (TP-DNA) were only 25% of the size of full-length DNA. The factor was eventually purified from uninfected Hela nuclear extracts and was termed nuclear factor II (NF-II). SDS PAGE analysis revealed NF-II to be a 30KDa complex of two proteins with molecular weights of 15KDa and 17KDa. These purified NF-II preparations contained no detectable nuclease, ATPase, RNA or DNA polymerase activities, but contained Type I topoisomerase activity. NF-II which is not a sequence specific DNA binding protein like NF-I and NF-III, was found not to be required for the initiation of DNA replication or even in the elongation of short templates. However, it was required for the synthesis of full-length DNA strands. NF-II's function could be substituted by the 100KDa eukaryotic topoisomerase I, suggesting that NF-II may be a different species of cellular topoisomerase or even a "core" subunit of topoisomerase I which has undergone proteolytic cleavage.

7.4. Factor pL.

Factor pL was first identified by Guggenheiner et al., (1984) who purified the 44KDa protein from uninfected Hela nuclear extracts on the basis that it was specifically required for the initiation of plasmids containing the adenovirus origin of replication. pL was found to have no effect on TP-DNA template replication, but was required for TP minus template

replication. The purified protein contains a 5'-3' exonuclease function which is thought to stimulate the initiation of adenovirus DNA replication by rendering the 3' end of the adenovirus genome single-stranded, allowing pTP-pol direct access to the 3' end. However, the use of partially single-stranded oligonucleotides as templates demonstrated that pL's function could be abolished (Kenny et al., 1988). This suggests that pL is unlikely to play a role in DNA replication in vivo, since it doesn't affect the in vitro DNA replication of TP containing templates.

8. Adenovirus type 4 DNA replication.

Early transfection studies demonstrated that plasmids containing an Ad.2 replication origin could be replicated efficiently when supported with either an Ad.2 or Ad.4 helper virus. However, plasmids containing Ad.4 origin sequences were only found to be efficiently replicated when co-transfected with Ad.4 helper virus genome.By comparison co-transfection with Ad.2 helper virus gave around a 20-fold reduction in replication (Hay et al., 1985b). Sequence analysis of the replication origins of Ad.2 and Ad.4 revealed that whilst the 1-18 "core" domain of the origin is identical in both serotypes, Ad.4 possesses an A/T rich domain in place of an NF-I binding site. Furthermore it was demonstrated that Ad.4, in contrast to Ad.2, requires only the terminal 18bp of the viral genome for fully efficient DNA replication in vivo.Subsequent in vitro replication studies confirmed the in vivo observations when it was demonstrated using a crude Ad.4 infected Hela cell extract that linearised plasmid containing only the terminal 18bp of an adenovirus ITR could support initiation of DNA replication (pTP-dCMP complex formation) in vitro as efficiently as a template containing a complete AD.4 ITR (Harris and Hay, 1988). Recently, a combination of more extensive sequence analysis (Temperley et al., 1991) and in vitro replication studies using a

purified Ad.4 infected Hela cell extract (Temperley and Hay, 1991) has identified two distinct regions within the adenovirus that are required for efficient DNA replication. Temperley et al., (1991) examined the effect of single base changes in positions 9 to 18 of the Ad.4 origin on DNA replication in vitro. Changes in the bases between 12 to 16 had little effect, whereas alterations at positions 9, 10, 11, 17 and 18 all reduced the efficiency of initiation of DNA replication by between 50 and 90%. It was concluded that the region from 9 to 18 bp contained two sets of bases (separated by 5base-pairs) essential for DNA replication. The region between 9-18bp is thought to be part of the recognition site on Ad.4 for the pTP-pol heterodimer. Strong proof for this theory has come from studies on Ad.2 when Temperley and Hay, (1992) demonstrated that the region between 8 to 17bp was the recognition site for the Ad.2 pTP-pol heterodimer. The basis of the difference in origin sequence requirements between Ad2/5 and Ad4 is that Ad4 appears to be able to replicate its DNA without the need for auxiliary stimulatory functions provided by the binding of NF-I and NF-III. Ad.4 does not possess an NF-I recognition site although it does have a strong consensus binding site for NF-III located at the same position as in the Ad2/5 origin of replication. However, it has been unequivocally demonstrated that neither cellular factor stimulates Ad4 DNA replication even if their cognate binding sites are present in a reconstituted origin of replication (Hay et al., 1988). Since it appears that NF-I and NF-III play auxiliary roles in Ad2/5 DNA replication, whereby the function of the viral replication proteins are enhanced or aided by their binding at the origin, the probability is that Ad.4 is able to replicate its DNA without them because its own replication proteins alone can perform the required functions. Ad.4 therefore potentially provides a more simplified system in which to

further study the molecular details of the mechanism of adenovirus DNA replication.

9. Simian Virus 40 DNA replication.

Studies on the DNA replication of SV40 have proven it to be an excellent model system for providing information on the mechanisms underlying cellular DNA replication.

The SV40 genome consists of a circular duplex DNA molecule of about 5000 base-pairs and contains a single origin of DNA replication. Replication takes place in the nucleus of the host cell where the genome is complexed with cellular histones to form a nucleoprotein complex (minichromosome) which is indistinguishable from cellular chromatin. Since SV40 only encodes a single viral protein involved in DNA replication, T-antigen (an 82KDa phosphoprotein), extensive use is made of the replication machinery in a manner which differs little from that of cellular DNA replication. In both SV40 and cellular DNA synthesis, initiation results in the creation of two replication forks that move bi-directionally from the origin. At each fork one of the two nascent strands (the leading strand) grows continuously, while the other, lagging strand grows discontinuously by joining together small 200bp segments of DNA (Okazaki fragments) that are independently initiated with RNA primers from DNA primase enzymes. Completion of replication occurs when two oppositely moving forks meet (reviewed; Hay and Russell, 1989, Fanning and Knippers, 1992).

Just as in the study of adenovirus DNA replication, the development of an efficient in vitro cell-free replication system in which SV40 DNA replication could be initiated and elongated, provided the opportunity for greatly accelerated progress in understanding the mechanisms involved in the replication process. This breakthrough came when Li and Kelly

(1984) purified an "active" soluble extract from SV40 infected cells that could support initiation and elongation of an exogeneously added DNA template containing the SV40 DNA origin of replication. The system was found to be absolutely dependent on the presence of the viral T-antigen protein and the origin region of the SV40 genome. An important dividend from the systems development has been the identification and characterisation of the components of the cellular machinery involved in SV40 DNA replication.

9.1. The origin of SV40 DNA replication.

The SV40 origin of replication is a 64bp segment of the viral genome that has been found to contain all the nucleotide sequence elements required for initiation of viral DNA replication in vitro and in vivo. Extensive mutational analysis of base substitutions has revealed that the origin is complex, consisting of three distinct sequence domains essential for origin function. It is composed of a central sequence element containing four copies of the pentameric sequence 5'-GAGGC-3' organised as a palindrome, and serves as the binding site for the viral T-antigen (T-ag) (Tegtmeyer et al., 1983 ; Stillman et al., 1985). The primary role of this site is to bind T-ag and position it in the proper location and orientation for its subsequent function in DNA replication. It has been demonstrated that T-antigen binds to this sequence in the absence of ATP forming a range of oligomeric structures from monomers up to tetramers (Mastrangelo et al., 1985). The other two domains of the "core" region are located on either side of the T-ag binding site. On one side there is a 17 base-pair A/T rich region which untwists upon ATP dependent T-ag binding. (Boroweic and Hurwitz, 1988a). On the other side of the site is a 10bp imperfect palindrome sequence known as the "early palindrome" (Deb et al., 1986). This sequence element contains an 8bp purine-

pyrimidine tract that opens upon T-ag binding (Borowiec and Hurwitz, 1988b). All three sequences are required for SV40 DNA replication and there is some evidence that the spacing between them may be critical (Parsons et al., 1990).

The 64bp "core" region may be sufficient to support initiation, but sequences outside of the "core" can have a profound effect on the efficiency of the process. Of even greater importance, in vivo, are two auxiliary sequence components which flank the SV40 origin, Aux-1 (another T-ag binding site, termed site I) and Aux-2 (contains three Sp1 binding sites and three T-ag-binding sites). Aux-1, depending on experimental conditions, can stimulate SV40 DNA replication up to 70-fold, whereas Aux-2 can stimulate it up to 100-fold (Guo et al., 1991 ; Guo and DePamphilis, 1992). Maximum stimulation of SV40 DNA replication in vitro requires the presence of both sequences which are thought to be involved in the unwinding of the "core" region. Deletion of Aux-1 and Aux-2 reduced the efficiency of origin specific DNA unwinding when either T-ag containing cell extracts or purified T-ag was used (Gutierrez et al., 1990). This data suggested that both Aux-1 and Aux-2 facilitate the T-ag dependent unwinding of the SV40 DNA origin. This is thought to be due to the T-antigen's ability to form protein-protein interactions with the proteins that bind at the Aux sequences, promoting DNA unwinding by stabilising the initial unwound replicative intermediate (reviewed in DePamphilis, 1993).

9.2. Initiation of SV40 DNA replication and bidirectional DNA synthesis:

T-antigen (T-ag).

Production of the SV40 large T-antigen is initiated soon after infection by the virus. The T-ag plays multiple roles during productive infection; it regulates the timing of the infection cycle, repressing transcription of its own gene, initiating viral DNA replication and stimulating the expression of viral capsid proteins. It also stimulates cell proliferation, cell transformation and the induction of tumours in animals (reviewed in Fanning and Knippers, 1992). Correspondingly, it has been shown to interact with cellular proteins implicated in cell growth control, such as the tumour suppressor p53 (Braithwaite et al, 1987) , the retinoblastoma suppressor gene product (De Caprio et al., 1988), replication factor A (Dornreiter et al, 1992) and the DNA polymerase α-primase complex (Smale and Tijian, 1986 ; Dorneiter et al, 1990).

The large T-ag protein has three main roles in SV40 DNA replication. Firstly, it binds as a double hexamer to specific sequences ("core") within the origin of replication, forming an ATP dependent initiation complex (Boroweic et al, 1990). This results in the structural distortion and local untwisting of the DNA at the origin. Secondly, it has an intrinsic DNA helicase activity which is required for bidirectional unwinding of the origin and extending unwinding during DNA synthesis (Dobson et al., 1987 ; Dean and Hurwitz, 1991). Last of all it guides the DNA polymerase α-primase complex into the unwound origin via specific protein-protein contacts with itself and RF-A. These interactions position the DNA polα-primase complex in an orientation suitable for subsequent full-length DNA synthesis (Collins et al, 1993).

Replication Factor A (RP-A).

RP-A is a heterotrimeric protein complex consisting of 70KDa, 34KDa and 11KDa subunits, and participates in the unwinding process as a result of its 70KDa subunits ability to unwind DNA strands and stabilise regions of ssDNA already created by T-ag and itself, (Georgaki, and Hubscher, 1993). The T-ag independent unwinding activity of the 70KDa subunit is thought to be facilitated through phosphorylation of the 32KDa RP-A subunit by the cdc2 family of kinases (Fotedar and Roberts, 1992). The importance of RP-A's contribution to SV40 DNA replication was confirmed when kinetic analysis on in vitro DNA replication studies demonstrated that the presence of RP-A led to the elimination of a 10-15 minute lag which usually preceded the start of DNA synthesis (Wobbe et al, 1986 ; Fairman and Stillman, 1988 ; Wold and Kelly, 1988). This indicated that unwinding of the origin of replication may be the rate-limiting step in initiating DNA synthesis and that RP-A is the only cellular protein known to be essential for this step. This preinitiation complex of T-ag and RP-A is then thought to be converted to an "initiation" complex by the recruitment of the cellular DNA polα-primase complex. This step is determined by specific protein-protein contacts between T-ag, RP-A and DNA polα-primase (Collins et al, 1993). The interactions between these proteins are thought to help in the suppression of unspecific priming events.

RP-A is also required during the elongation of SV40 DNA, to stimulate the activity of both the cellular DNA polα-primase complex (Kenny et al., 1989 ; Tsurimoto and Stillman, 1989a, 1991). RP-A stimulates DNA polα-primase activity through direct physical interactions with the 70KDa and 32KDa subunits (Fang and Newport, 1994). These interactions are highly specific as both procaryotic and viral

single-stranded proteins cannot be substituted for RP-A in complete replication reactions (Kenny et al, 1988) This specific interaction between ssDNA binding proteins and polymerases can be seen throughout procaryotic (E.coli and Bacteriophage T4 and T7) and eukaryotic (Adenovirus and Herpes Simplex virus) viral DNA replication systems. RP-A (via its 70KDa subunit) has also been shown to bind to and stimulate the activity of the cellular DNA polymerase δ. The stimulatory effect is complex as it requires the presence of at least two other accessory proteins: proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) ,which with RP-A are needed for optimal stimulation (Tsurimoto and Stillman., 1989a, 1989b ; Kenny et al., 1989). DNA polymerase δ, unlike DNA polymerase α, is less dependent on RP-A originating from the same organism. Tsurimoto et al., (1990) demonstrated that SSB's from both E.coli and adenovirus could substitute for RP-A derived from the same tissue.

Cellular DNA polymerases and processitivity factors.

Early in vivo SV40 DNA replication studies using inhibitors implied that cellular DNA polymerase α was involved in the replication process (Krokan et al., 1979). Several lines of evidence suggested that this was also the case in vitro. Initial experiments (Ariga and Sugano, 1983 ; Li and Kelly, 1984) showed that replication in vitro was inhibited by aphidicolin, an inhibitor of DNA polymerase α, and N-ethylmaleimide, an inhibitor of polymerase α and β. In contrast dTTP, an inhibitor of polymerases β and γ, had no effect on replication. When the polymerase was purified from Hela cell extracts it was found to be tightly associated with a DNA primase activity (Murakami et al., 1986). Both of these activities are absolutely required for SV40 DNA replication in vitro (Murakami et al., 1986). The DNA polα-primase complex which has

been implicated as the lagging-strand polymerase in SV40 DNA replication (Collins and Kelly, 1991 ; Murakami and Hurwitz, 1993), is a four subunit enzyme that exhibits both DNA polymerase and primase functions. These activities were first found in the DNA polα-primase complex of Drosophilia melanogaster (Cotterill et al., 1987a, 1987b). DNA polα-primase influences SV40 DNA replication through the different functions its subunits contain. The largest of these is the 180KDa subunit A. This subunit contains the DNA polymerase catalytic activity and forms a physical interaction with the viral T-ag protein (Dornreiter et al., 1990, 1993). Early in vitro SV40 DNA replication assays revealed that RP-A associated with both the DNA pol α-primase complex and the T-antigen (Dornreiter et al., 1992) in a trimeric complex. The interaction between T-ag and the DNA pol α-primase was found to be significantly important as the physical coupling of these two components of the replication apparatus increases the efficiency with which new DNA chains are initiated (Collins and Kelly, 1991 ; Erdile et al., 1991 ; Melendy and Stillman, 1993). Absence of any one of these components leads to the abolishing of SV40 DNA replication.

The B-subunit(s) of polymerase α was initially purified and sequenced from Drosophilia melanogaster (Conaway and Lehman, 1982). Only recently has the B-subunit of human polymerase α been cloned and expressed in large enough amounts to use in characterisation studies. Collins et al., (1993) demonstrated that purified human B-subunit protein was capable of binding to the viral T-antigen as well as subunit A (Catalytic domain) of the DNA polymerase α. Addition of exogenous subunit B protein was found to greatly enhance the formation of the complex between the catalytic domain of DNA polymerase α (subunit A) and the viral T-antigen. These physical interactions were shown to be functionally important when it was demonstrated that T-ag stimulated the

activity of the DNA polymerase α catalytic subunit on model templates only in the presence of subunit B. This suggested that subunit B was acting as a molecular tether whose function was to couple the DNA polα-primase complex to the unwinding apparatus. This would increase the efficiency of initiation of the first DNA chains at the origin and facilitate the subsequent priming and synthesis of DNA chains on the lagging strand template by holding the polymerase within the domain of the DNA template after it has dissociated from the terminus of a newly synthesised DNA strand (Wang, 1991 ; Collins et al, 1993).

All DNA polymerases require a 3'-hydroxyl terminus of a pre-existing primer for polymerisation (reviewed in Kornberg, 1980). To date DNA polymerase α is the only eukaryotic polymerase with a tightly associated primase. The primase activity of the DNA polα-primase complex resides in its two smallest subunits C (53KDa) and D (48KDa) (Kaguni et al., 1983 ; Tseng et al., 1983 ; Cotterill et al., 1987b). The role of the primase is centred on the lagging strand template where it synthesises short base-paired RNA primer strands. These are then "filled in" by the catalytic subunit (A) of DNA polymerase α during elongation (Roth, 1987).

A second cellular DNA polymerase, DNA polymerase δ, is involved in the synthesis of SV40 DNA. It consists of a 125KDa catalytic polypeptide with an associated 48KDa subunit (function unknown) and possess a 3'-5' exonuclease activity (Byrnes et al., 1976). Its role was initially suggested from work on the identification and purification of the 36KDa proliferating cell nuclear antigen factor (PCNA), which was found to be essential for SV40 DNA replication in vitro (Mathews et al., 1984 ; Prelich et al., 1987a). Further in vitro studies revealed that the low processitivity of DNA polymerase δ on long single-stranded templates could be increased by the addition of exogenous PCNA (et al., 1987b).

This implied that both the polymerase and PCNA had essential roles in SV40 DNA replication.

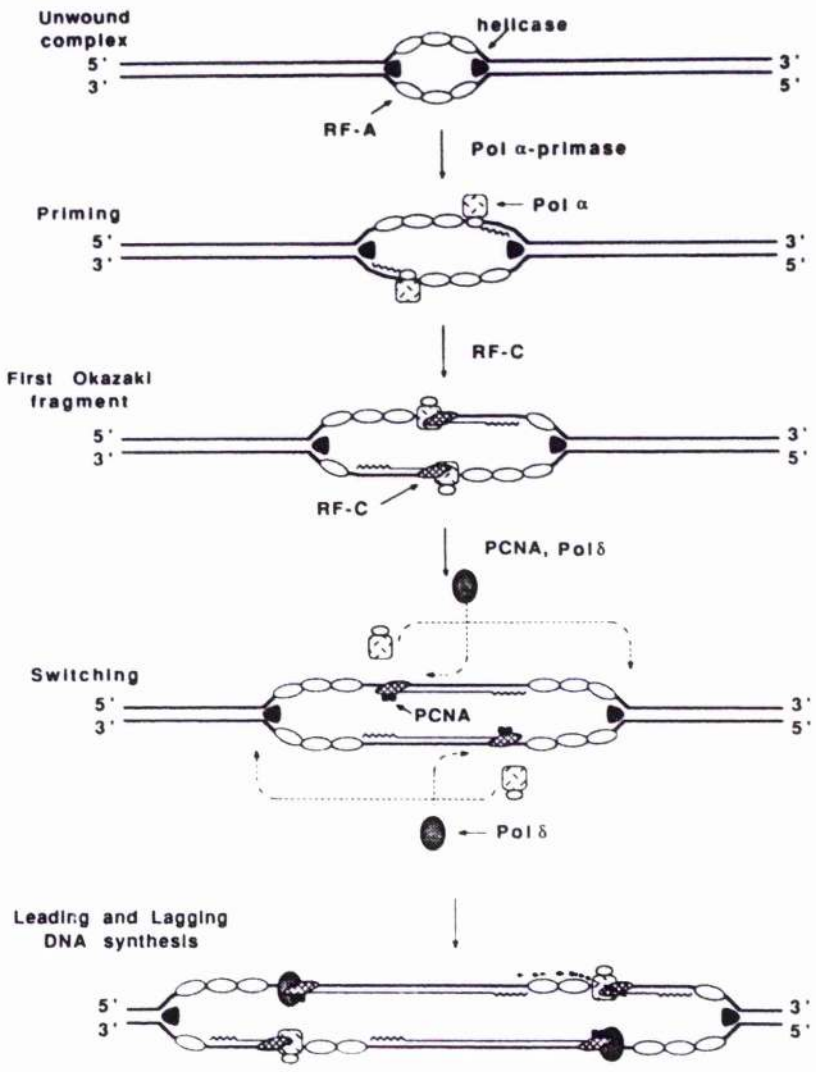
PCNA's role in SV40 DNA replication was revealed in an in depth study which compared the structure of in vitro SV40 replication products formed both in the presence and absence of PCNA (Prelich and Stillman, 1988). The data from these studies demonstrated that PCNA was not required during the initiation of SV40 DNA replication and in the formation of primary nascent strands at the replication origin. However, subsequent elongation in the absence of PCNA resulted in the shutdown of leading strand synthesis and the production of short DNA products,which dissociated from the template. This implied that PCNA was required for co-ordinated leading and lagging strand synthesis.

A second factor called replication factor C (RF-C) was also identified and found to be associated with only the elongation phase of SV40 DNA replication. Its omission from an in vitro DNA replication reaction had the same effect on the SV40 DNA replicative products as the omission of PCNA (Tsurimoto and Stillman, 1989b). Further investigations revealed that both PCNA and RF-C recognise and bind to the lagging strand polymerase complex at the replication origin forming a "primer recognition" complex (Tsurimoto et al., 1990). This complex is thought to play a crucial role in blocking the DNA polα-primase complex and in initiating leading strand synthesis from the nascent DNA fragment synthesised by the lagging strand DNA polymerase complex (reviewed in Bambara and Jessee, 1991). Recently, a third polymerase species (Pol ε) has been found and is thought to be involved in the elongation of the lagging strand. However, its existance is still controversial (reviewed in Bambara and Jessee, 1991).

Using the type of assay where SV40 DNA replication is reconstituted entirely from purified proteins which can be excluded or omitted,

Figure 4. Model for initiating leading- and lagging-strand synthesis with two DNA polymerases.

The first line of the figure shows the unwound complex stage, followed by five subsequent stages: 1. primer RNA synthesis with primase (Priming), 2. first Okazaki fragment synthesis with polymerase α, 3. switching of DNA polymerases at the 3' end of this first Okazaki fragment, and finally, 4. leading- and 5. lagging-strand synthesis by polymerase δ and α. Factors involved are DNA helicase (T antigen in the case of SV40), RF-A, polymeraseα-primase, RF-C, PCNA and polymerase δ. The factors are introduced according to their entry into the reaction. The roles of the topoisomerases are not indicated (taken from Wang, 1991).


Unwound complex
5'
3'
helicase
3'
5'
RF-A
Pol α-primase
Priming
Pol α
RF-C
First Okazaki fragment
RF-C
PCNA, Polδ
Switching
PCNA
Polδ
Leading and Lagging DNA synthesis

Tsurimoto and Stillman, (1990) have proposed a "polymerase switching" model for the initiation of bidirectional DNA replication and subsequent elongation of leading and lagging strand strands.

"Polymerase switching" model for the mechanism of SV40 DNA replication.

The model (see figure4) proposes that DNA polymerase α–primase primer synthesis ensues from the unwound origin site generated by T-antigen (helicase function), RF-A and DNA topoisomerase action. Addition of both RP-A and RF-C facilitates DNA pol α-primase priming and lagging strand synthesis to some degree, based on their known stimulation of DNA pol α-primase activity. PCNA addition to the nascent Okazaki fragment at the origin induces the ATP-independent binding of RF-C and DNA polmerase δ to the 3' terminus, displacing DNA polymerase α for another cycle of Okazaki fragment synthesis on the lagging strand. Leading strand synthesis is then mediated by the RF-C / PCNA / DNA polymerase δ complex. The dissociated DNA pol α-primase complex then returns to the next priming site on the lagging strand by interaction with T-antigen and continues to copy the lagging strand template discontinuously.

10. Herpes Simplex virus DNA replication.

The herpesviruses are a large group of DNA containing viruses which have been isolated from humans and many different animal species. The most extensively characterised of all the groups members are human HSV-1 and HSV-2. Like all herpesviruses HSV can maintain a latent infection (its genome being harboured in the neurons of sensory ganglia) and replicate lytically in the epithelial cells of an infected individual as

well as in a wide variety of cultured mammalian cells. The HSV-1 genome contains a linear double-stranded DNA of approximately 152kbp, which has been completely sequenced and found to encode over 70 distinct proteins (McGeoch et al., 1988) including all those required for the replication of its genome. The HSV-1 genome consists of two components: a long (L or U_L) and short (S or U_S) unique sequence each of which is flanked by inverted terminal repeats. During the course of viral replication both L and S invert relative to each other via a recombination mechanism leading to reorganisation, or isomerisation, of the genome. Since both the L and S sequence components can be in one of two orientations, purified replicated viral DNA consists of an equimolar population of four possible isomers soon after infection (Jenkins and Roizman, 1986).

To date no cell-free system for HSV DNA replication has been described, therefore most of our present knowledge has been derived from in vivo studies on replicative intermediates. These intermediates were found to have two characteristics; firstly, replicating viral DNA pulsed-labelled with [^{3}H] thymidine has a higher sedimentation coefficient than unit length viral DNA (Jacob and Roizman, 1977) and secondly that the same DNA is "endless", i.e. the molecular termini of mature viral DNA are fused together forming concatmer structures (Jacob et al, 1979). On the basis of these in vivo observations it was proposed that parental HSV DNA was circularised shortly after entry into the host cell, via complementary single nucleotide 3' overhangs at the genome termini, with subsequent replication probably taking place by a rolling circle mechanism, generating linear progeny genomes as linear concatamers of tandemnly repeated virus genomes (Jacob et al., 1979).

10.1. The HSV origins of DNA replication.

The HSV-1 genome has three origins of replication: two identical copies of Ori_S located within the repeated elements of the unique short segment of the viral genome and a single copy of Ori_L near the centre of the unique long segment of the viral genome. The functional significance of three separate origins of replication in the HSV genome is not clear. Mutant viruses lacking either one copy of Ori_S or Ori_L have been isolated and have no obvious defect in growth (Longnecker and Roizman, 1986). Based on the observation that transfected plasmids containing viral origins of replication are amplified in HSV infected cells, the sequence requirements for origin function were defined. The most extensive of these studies was carried out on plasmids containing Ori_S. Minimally, Ori_S is comprised of a 75bp segment of sequence containing a 44bp palindromic sequence of which the central 18bp are composed entirely of A/T base-pairs (Stow and McMonagle, 1983). The termini of the 44bp palindrome contains a 10bp sequence composed of overlapping inverted partial repeats. These 10bp sequences are homologous and are termed Box I and Box II. In addition, a third 40bp homologous partial inverted repeat , Box III, exists and is located to the left of the palindrome and is also required for Ori_S function.The minimum sequences required for the function of Ori_S correspond well to the region of highest similarity with Ori_L. However, Ori_L does contain a more extensive palindromic region of 144bp (Weller et al, 1985). Mutational analysis on the Ori_S sequence revealed that deletion of the central A/T rich region in the palindrome completely abolished DNA replication, as did replacement of the central A/T region with an equal number of GC base-pairs (Lockshon and Galloway, 1988). Both arms of the palindrome contain a binding site for the HSV origin binding protein, a product of the UL9 virus gene (Weir et

al., 1989). The role of this protein in DNA replication will be discussed in the following section. There is also evidence that regions of sequence flanking the core region have a modest effect on the extent of DNA replication, when measured in a plasmid amplification assay (Stow et al., 1982b ; Stow and McMonagle, 1983). However, more recent evidence by Su and Knipe (1987) found no evidence suggesting that these upstream regulatory elements had any direct effect on origin function.

10.2. HSV genes required for DNA replication.

For many years the use of virus mutants (usually temperature sensitive mutants) has provided the major experimental approach to determining whether specific viral gene products play a role in HSV DNA replication. Initial genetic studies were carried out on a large number of conditionally lethal mutants of HSV, found to be defective in DNA synthesis. Complementation studies revealed that mutants with defects directly affecting DNA replication fall into seven groups (Challberg, 1986 ; Wu et al., 1988). The complete set of viral genes that are required for viral DNA replication were identified by means of a transient complementation assay, in which cloned segments of HSV genome DNA were tested for their ability to support the replication of a co-transfected plasmid containing the HSV origin of replication (Challberg, 1986). The role of individual HSV DNA replication genes has also been examined using the recombinant baculovirus expression system. Infection of insect cells with baculovirus recombinants capable of expressing all the HSV-1 DNA replication proteins resulted in the viral origin dependent amplification of a transfected test plasmid (Stow, 1992). As mentioned above, early genetic studies on virus mutants revealed that seven HSV viral gene products were both necessary and sufficient for the replication of origin dependent DNA synthesis. Biochemical activities have now been

ascribed to each of these proteins (McGeoch et al., 1988 ; Wu et al., 1988). The seven genes encode: a highly processive heterodimeric DNA polymerase (Purifoy et al, 1977 ; Crute and Lehman, 1989), a heterotrimeric helicase/primase (Crute et al, 1988, 1989), a single-stranded DNA binding protein (Weller et al, 1983) and an origin-binding protein (Elias et al, 1986 ; Olivo et al, 1988). The roles of each of these proteins in HSV DNA replication will be discussed individually.

HSV Origin Binding Protein.

(Gene product: UL9)

The origin binding protein was initially identified in extracts of HSV infected cells by the its ability to specifically bind to the HSV origins of replication. The protein termed UL9, was purified to homogeneity by DNA recognition site chromatography and shown to have a molecular weight of 83KDa by SDS PAGE (Elias et al, 1986 ; Elias and Lehman, 1988). DNase I footprint analysis of the purified protein revealed that it bound to and protected two nearly identical sites (termed Box I and Box II) present in each arm of the palindrome region in Oris (Olivo et al, 1988). Methylation interference experiments combined with binding site affinity assays revealed that the protein bound to the recognition sequence GT(T/G)CG, contained within a 10 base-pair consensus sequence present in Boxes I and II. This recognition sequence is contained twice within the 10bp consensus sequence, as inverted repeats. Furthermore, filter binding studies with single strand oligonucleotides corresponding to Boxes I and II demonstrated that UL9 bound to the left arm sequence (Box I) of the palindrome with an affinity 5-10 times greater than the binding site on the right arm (Box II). However, while there is general agreement that the left arm of the palindrome and its A/T rich central region are essential for Oris function, there are conflicting

results on the requirement for the right arm sequence. Deb and Doelberg, (1988, 1989) have reported that a plasmid lacking the right arm Oris can replicate equally as well as a control plasmid containing the intact origin. Additional support for this view came from work by Stow and Davison, (1986) on the related Varicella zoster virus (VZV). Their results demonstrated that HSV cells could support the replication of plasmids containing the VZV origin of replication, which only contains sequences homologous to the left arm and A/T rich regions of the HSV Oris palindrome. However, more recently combined mutational and binding site studies by Weir and Stow, (1990) have demonstrated that both sets of sequence are required for efficient DNA binding and origin function. The results from these recent studies suggest that there is some form of cooperative interaction between the UL9 proteins bound at the two binding sites. Direct evidence for this theory came from in vitro mutational studies investigating the effect of insertions into the central A/T rich region situated between the two UL9 binding sites. These studies demonstrated that the insertion of n additional A/T base-pairs into the A/T rich domain had a periodic effect on the efficiency of Oris in DNA replication. As n was increased from 0 to 8, replication was seen to first decrease to a minimum at n=3, then rise to a maximum at n=5, before decreasing again when n was greater than 6 (Lockshon and Galloway, 1988). This strongly suggests that a strict spatial constraint on the positioning of the UL9 binding sites exists which may be necessary to accommodate UL9-UL9 interactions critical for HSV origin function.

Analysis on the primary amino acid sequence of the UL9 protein has revealed the existence of two leucine zipper binding motifs; one near the N-terminus which has been shown to be required for origin cooperative binding (Elias et al, 1992) and the other closer to the C-terminus of the protein which seems to be involved in protein-protein

interactions with other proteins involved in origin-specific initiation of HSV DNA replication. The UL9 protein is homodimeric and possesses both DNA-dependent nucleoside triphosphatase and DNA helicase activities (Dobson and Lehman, 1993 ; Boehmer et al, 1993). These enzymatic activities, along with its capacity to bind specifically origin DNA, are reminiscent of the SV40 T-antigen initiator protein. In the presence of ATP, T-antigen assembles into two double hexamers that untwist the SV40 DNA origin as a prelude to initiating DNA synthesis. In contrast, binding of the UL9 protein to Oris does not appear to be affected by ATP. Furthermore, it has recently been reported that the Herpes encoded single-stranded DNA-binding protein ICP8 can specifically stimulate the DNA helicase activity of the UL9 protein through a direct protein-protein interaction (Boehmer and Lehman, 1993a). Again this is reminiscent of SV40, with T-ag and RF-A substituting for UL9 and ICP8.

HSV Single-Stranded DNA Binding Protein ICP8.

(Gene product: UL29)

ICP8 (infected cell polypeptide 8) was initially recognised several years ago as an abundant HSV-induced 128KDa protein present in infected cells (Bayliss et al, 1975). Early genetic studies using HSV temperature sensitive virus mutants revealed that the protein was absolutely required for viral DNA replication (Conley et al, 1981 ; Weller et al, 1983). This led to the reasonable assumption that the function of ICP8 was analogous to a group of replication proteins, e.g. E.coli SSB, T4 gene 32, RF-A, Ad.DBP, etc. known as single-stranded DNA binding proteins. Their primary function in DNA replication is to bind to single-stranded DNA formed at the replication fork by the helicase dependent unwinding of the parental strand in order to facilitate the use of these strands as templates

for elongation by a replicative nucleoprotein complex. ICP8 has many of the characteristic properties associated with the SSB's: it binds to single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) and RNA in vitro (reviewed in Chase and Williams, 1986 ; Stuiver, 1993) with at least a 5-fold preference for ssDNA over dsDNA; binding of ICP8 to ssDNA is cooperative at saturating concentrations and is independent of sequence. Using both site-directed mutagenesis and protease digestion assays the DNA binding region of ICP8 was mapped to a region spanning amino acids 564-869 (Leinbach and Heath, 1988 ; Wang and Hall, 1990). Further sequence analysis revealed that residues 499-512 within this region conformed to the consensus sequence for a "zinc finger" DNA binding motif (Gupte et al, 1991). Another consequence of DNA binding by SSB's is their ability to regulate gene expression. Analysis of viruses containing mutant ICP8 revealed an accumulation of mRNA's from both early and late viral genes, indicating that ICP8 does plays a direct part in the regulation of HSV transcription (Godowski and Knipe, 1985 ; Gao and Knipe, 1991). As mentioned above, SSB's have the ability to assist in DNA synthesis by stimulating the proteins present in a viral replisome. ICP8 has been shown to stimulate the activities of all of the HSV viral gene products. Its interaction with the other viral products will be discussed individually:

ICP8-HSV DNA polymerase interaction.

Purified ICP8 has been shown to have a stimulatory effect on the HSV DNA polymerase activity in vitro using either primed ssDNA (activated DNA) and dsDNA as templates (Ruyechan and Weir, 1984 ; Hernandez and Lehman, 1990). It has also been found to stimulate the intrinsic 3'-5' exonuclease activity of the polymerase(O'Donnell et al, 1987a). Evidence for the stimulatory nature of ICP8 has also come from mutational studies

with HSV-2 ICP8 temperature sensitive mutants. HSV DNA polymerase activity was found to be reduced in extracts from cells infected with ICP8 temperature sensitive mutants at the non-permissive temperature. Additionally, some of the viral mutations were found to increase HSV DNA polymerase sensitivity to inhibitors like aphidicolin (Chiou et al, 1985). This is reminiscent of the suspected Ad.DBP-Ad.DNA polymerase interaction in the adenovirus system (Mul and Van der Vliet, 1993). The isolation of monoclonal antibodies against purified ICP8 and HSV DNA polymerase has produced detailed analysis on both of their intracellular localisation during infection (Bush et al, 1991). In vivo Immunofluorescence with these antibodies demonstrated that both ICP8 and HSV DNA pol. colocalised in distinct cellular structures known as prereplicative sites and replication compartments. The localisation of HSV DNA polymerase to the nucleus was found to be independent of ICP8, but in its absence remained diffusely distributed throughout the cell nucleus. This suggests that the correct intranuclear distribution of the viral polymerase requires the presence of ICP8. The colocalisation of a viral SSB and viral DNA polymerase in distinct replication compartments has been shown in other viral replication systems, including adenovirus (Bosher et al., 1992). So clearly, similar compartmentalised functions exist throughout eukaryotes. No evidence supporting a direct interaction between ICP8 and the HSV DNA polymerase has been found to date. As with the adenovirus system, the SSB (ICP8) is thought to probably affect DNA polymerase activity through its ability to distort the secondary structure of the DNA it binds to.

ICP8-UL9 Interaction.

ICP8 can stimulate both the DNA-dependent ATPase and DNA helicase activities (Dobson and Lehman, 1993) of the origin binding protein, UL9.

The stimulatory effect was specific for ICP8; neither E.coli SSB or Replication Factor A (RP-A) were able to substitute its function. These results suggested that there may be a direct interaction between ICP8 and UL9. ICP8 is believed to aide the UL9 protein in the physical separation of the DNA duplex, as it contains a helix-destabilising property similar to that of RP-A (Boehmer and Lehman, 1993b). ICP8's ability to bind ssDNA preferentially may also assist in this process by preventing the non-productive binding of UL9 to the single-stranded regions of the DNA substrate. A direct physical interaction between ICP8 and UL9 has recently been reported. Using protein affinity chromatography, Boehmer et al., (1993) demonstrated that baculovirus purified UL9 could bind to immobilised ICP8 and vice versa. The specific site of interaction has been localised to the 37KDa C-terminal domain of the UL9 protein, which has recently been shown to contain a leucine zipper binding motif (Deb and Deb, 1991). It is currently thought that the UL9-ICP8 interaction is mediated through this leucine zipper structure.

ICP8-Helicase/Primase Interaction.

ICP8 is required for the complete unwinding of duplex DNA by the helicase/primase complex. In vitro DNA helicase assays by Crute and Lehman, (1991) demonstrated that both ICP8 and ATP were required to produce fully unwound DNA template. No biochemical evidence for a direct interaction exists. As in the case of the ICP8-UL9 interaction, it is believed that ICP8 may serve to stabilise regions of ssDNA generated by the helicase.

HSV DNA polymerase + Processitivity subunit complex.

The HSV DNA polymerase was initially identified as a novel polymerase activity from HSV-infected cell extracts. The activity is readily

distinguishable from host cell DNA polymerases on the basis of its sensitivity to various inhibitors (e.g. aphidicolin and acylovir) and its stimulation, rather than inhibition, by moderate salt concentrations (Powell and Purifoy, 1977). Further analysis on both temperature sensitive and drug resistant mutants clearly demonstrated that the polymerase was virally encoded. Careful mapping of the drug resistant mutants revealed that the HSV DNA polymerase contained domains of highly conserved amino acids important for its function (Larder et al., 1987 ; Gibbs et al., 1988) e.g. the C-terminal amino acids 397-961 contain a substantial number of these mutations, indicting that it may contain the nucleotide binding site of the polymerase. Interestingly, these domains seem to have homology with domains in the amino acid sequence of many DNA polymerases, both prokaryotic and eukaryotic (see later in this section). This idea has been reinforced recently by data from limited proteolysis mapping and fluorescence quenching studies on the polymerase (Weisshart et al, 1993). These biophysical techniques have revealed that the site in the centre of HSV DNA polymerase whose accessibility increases upon DNA binding is near or within the putative C-terminal nucleotide binding site between a.a.397-961. To date the 136KDa HSV DNA polymerase has been purified from both HSV infected cell extracts and HSV recombinant baculovirus cell extracts.

Initial purification from HSV infected extracts revealed that the polymerase co-purified with a 62KDa protein which was subsequently shown to be the product of the UL42 gene, one of the genes found to be essential for DNA synthesis (Powell and Purifoy, 1977 ; Vaughan et al., 1985). Further investigations revealed that this protein was, in fact, a processitivity factor that existed with the polymerase in a dimeric complex (Hernandez and Lehman, 1990). UL42 is required, along with ICP8, for the enhancement of processitivity of deoxynucleotide

polymerisation by the HSV DNA polymerase subunit. UL42 can therefore be thought of as resembling the β-subunit of the E.coli DNA polymerase III holoenzyme. Biochemical analysis on UL42 free HSV DNA polymerase has shown it to be a trifunctional complex consisting of DNA polymerase, 3'-5' exonuclease and 5'-3' RNase H activities. The polymerase, despite its slow rate of DNA synthesis, is highly processive (Crute and Lehman, 1989 ; O'Donnell et al, 1987a), no doubt due to the presence of UL42 . Additionally, its rate of synthesis can be increased substantially in the presence of saturating concentrations of the ICP8. However, unlike in the case of the adenovirus SSB, Ad.DBP, ICP8's effect could be fully substituted by the E.coli SSB. There is indirect evidence from in vitro DNA-binding (O'Donnell et al, 1987b) and in vivo immunofluorescence (Bush et al, 1991) studies, that ICP8 can form a complex with the HSV DNA polymerase. To date no direct biochemical evidence exists that supports a physical interaction between the two. However, we can hypothesise that the increase in the rate of nucleotide incorporation observed in the presence of ICP8 may be due to its ability to hold ssDNA in a configuration which increases the efficiency of the viral DNA's movement along the DNA chain.

As mentioned above, the HSV DNA polymerase also contains a 3'-5' exonuclease and 5'-3' RNase H activity (Crute and Lehman, 1989). Both of these activities copurify with the DNA polymerisation function situated in the 136KDa polypeptide of the UL42-DNA polymerase complex. The 3'-5' exonuclease activity is thought to have a proofreading function which it uses to increase the fidelity of DNA synthesis. The 5'-3' RNase H activity is probably involved in the removal of RNA primers that initiate the synthesis on the lagging strand at a replication fork during HSV DNA replication.

Helicase-Primase complex.

(Gene products: UL5; UL8 and UL52).

The helicase/primase complex was initially identified by its DNA helicase activity which was induced upon HSV infection of cells (Crute et al, 1988, 1989). It was purified to homogeneity and found to consist of three subunits with molecular weights of 120KDa, 97KDa and 70KDa. Immunochemical analysis of the enzyme revealed that the three subunits were products of the HSV UL52, UL5 and UL8 genes (Crute and Lehman, 1991). The DNA primase activity co-purified with the DNA helicase activity during purification. in vitro characterisation of the enzyme complex demonstrated that it could not fully unwind partial DNA duplexes without the presence of the HSV SSB, ICP8. These results led to the suggestion that ICP8 may serve to stabilise ssDNA generated as a consequence of the helicase activity in the complex. Alternatively, ICP8's function could be to localise the helicase-primase complex to the replication fork by preventing non-productive interactions with the newly synthesised ssDNA chains.

Although no cell-free system for HSV origin-dependent DNA replication exists, one can hypothesise about the roles of the seven viral gene products in DNA replication, when one compares their respective biochemical properties with replication proteins involved in other eukaryotic viral DNA replication systems.

10.3. A model for HSV DNA replication.

HSV DNA replication probably initiates when the UL9 protein assembles into a multimeric structure at the HSV origin, resulting in the untwisting of the central A/T rich region into a recognisable "open" complex. This "open" complex could be facilitated by the coupled DNA-dependent ATPase and DNA helicase activities of the UL9 protein, in a similar

manner to the viral T-antigen of SV40. Complex formation would also require the presence of the single-stranded binding protein, ICP8, as it has been shown to stimulate the activity of the origin binding protein UL9. It also contains a helix-destabilising activity which could help in both the opening of duplex DNA and the stabilisation of regions of newly created ssDNA. This "open" complex would then require the presence of proteins involved in the initiation and elongation of the HSV genome. The HSV DNA polymerase and helicase/primase are vital for these processes. They either recognise and bind to the "open" complex or are guided to it by ICP8, which is suspected of forming complexes with both. The assembly of the seven viral protein products at the origin can be seen as forming a replisome (as in E.coli), which with the aid of as yet unknown cellular factors, replicates the HSV DNA genome.

11. Eschericia coli chromosomal DNA replication.

Over the last 20 years three general methods have been utilised to help identify E.coli DNA replication functions: isolation of mutants; sensitivity of replication to chemical inhibitors and the biochemical isolation of proteins required for DNA replication in vitro.

E.coli DNA replication initiates (see figure5) bidirectionally at a specific sequence (Ori C) located at about 84 minutes on the genetic map (Yasuda and Hirota, 1977). The origin itself was originally identified by both biochemical and gene dosage experiments. OriC was subsequently cloned and found to support the replication of plasmids lacking a functional origin (Von Meyenburg et al, 1979).The minimal site required for replication on plasmids in vitro is a 245bp sequence (Oka et al, 1980) which is found to be highly conserved among enterobacteria. OriC has several important sequence elements; there are four 9bp sites, denoted R1-R4 that are highly conserved and arranged into two inverted repeats.

This sequence known as Dna Box A was shown to be the binding site for Dna A, the E.coli initiator protein (Fuller et al, 1984 ; Matusi et al, 1985). To the left of the Dna A boxes are three direct repeats of a 13bp sequence (13mers) that are rich in A and T nucleotides (Bramhill and Kornberg, 1988a). DNA strand separation of the origin is initiated at these sites (Bramhill and Kornberg, 1988b). The origin also contains specific binding sites for two small double-stranded DNA binding proteins, IHF and FIS. These proteins along with the non-specific binding protein HU ("histone-like") function as accessory factors during the assembly of the many different protein-DNA complexes at the origin (Schmid et al., 1990).

The major breakthrough in our understanding of the mechanism of initiation at OriC was elucidated by the development of a crude extract system that could synthesise DNA specifically on OriC containing plasmids in vitro (Fuller et al, 1981). Subsequent efforts have now outlined the steps necessary for initiation. The central and first step of initiation from OriC is the binding of Dna A to the origin. This binding reaction is highly cooperative and results in 10-20 monomers of Dna A forming a complex with the DNA (Fuller et al, 1984). Dna A, which has an ATPase activity, then binds ATP and becomes active for initiation. The DnaA-ATP complex then promotes opening of the DNA in the region of the 13mers (Bramhill and Kornberg, 1988a, 1988b). These "open" DNA complexes were detected through the sensitivity of the DNA in this region to cleavage by a single-stranded endonuclease. Opening of these complexes was demonstrated to occur sequentially from the rightmost 13mer leftward and required the presence of the proteins HU or IHF (Yung and Kornberg, 1989). After the origin is opened, DnaB (a 5'-3' DNA helicase) is transferred to the exposed ssDNA as a DnaB-DnaC complex. DnaC functions as an escort protein as DnaB has a low

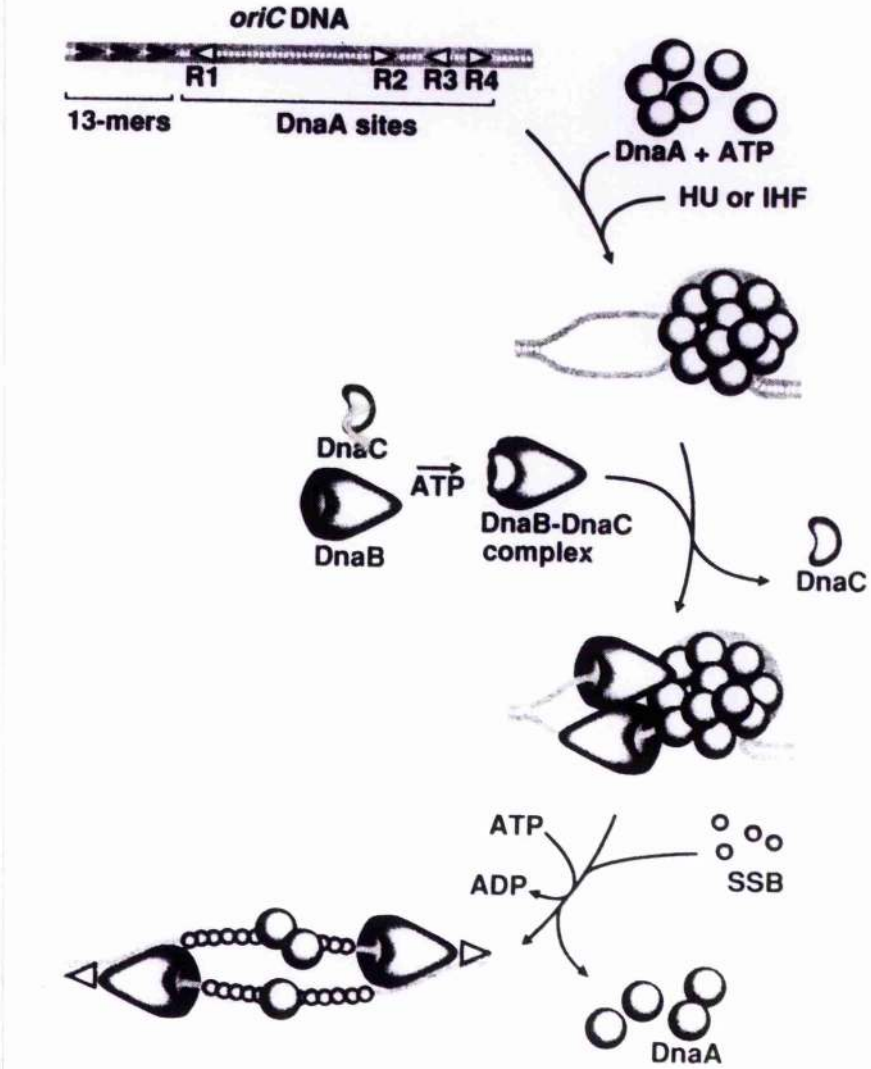


Figure 5. Model of the initiation of replication at OriC

The diagram does not suggest the stoichiometry of the proteins in the postulated complexes or the molecular structure of the individual proteins (taken from Baker and Wickner, 1993).

affinity for both single and double-stranded DNA in its absence. DnaA is also involved in bringing DnaB to the origin.It has been proposed that DnaC recognises DnaA at the origin and facilitates the binding of DnaB to the origin (Funnell et al., 1987). A direct role for DnaA and DnaC in loading DnaB is implied by the observation that DnaA also promotes the transfer of DnaB to any ssDNA, in a reaction that also requires DnaC (Wahle et al., 1989a). However, electron microscopy of the initiation complexes formed at OriC and tagged with gold-conjugated antibodies demonstrated the presence of DnaB, but failed to detect DnaC (Funnell et al., 1987 ; Wahle et al., 1989b). This data along with other studies indicate that DnaC is in fact released from its complex with DnaB during transfer onto the template. It is believed that the release of DnaC activates DnaB for later steps, i.e. elongation (Allen et al., 1991). The next stage in initiation involves the unwinding of the DNA from the open OriC sequence by DnaB's helicase activity (Baker et al., 1987). This requires the presence of ATP, DNA gyrase (a Type I topoisomerase) and E.coli SSB. Once unwinding by DnaB has begun, DnaG primase, through its interaction with DnaB, syntheses primers which are then elongated by the replicative machinery which has been brought to the template by DnaB. In the following section the characteristics of DnaB, DnaG primase, DNA polymerase III and E.coli SSB will be discussed, as they are principally responsible for full-length DNA replication in E.coli.

11.1. Elongation of E.coli chromosomal DNA replication.

Principal proteins involved in elongation are:

DnaB

Both biochemical and genetic analysis indicate that the 52KDa DnaB protein is the major helicase involved in E.coli DNA replication. Its helicase activity was first demonstrated in vitro by LeBowitz and

McMacken (1986) through the protein's ability to displace a ssDNA fragment from a partially duplex substrate in a reaction that required dNTP hydrolysis. By taking advantage of its substrate preference for dNTP hydrolysis, DnaB was shown to be the helicase involved in the unwinding (in vitro) of the OriC DNA template. The DNA helicase activity of DnaB has a 5'-3' polarity, placing it on the lagging strand template during replication, ahead of the leading strand polymerase. DnaB has also been shown to have a functional interaction with both the DnaG primase and DNA polymerase III holoenzyme proteins.

DnaG primase.

The 64KDa DnaG protein was identified as a primase based on the resolution and reconstitution of the enzymes required for the conversion of bacteriophage G4 ssDNA to its replicative form in vitro (Bouche et al., 1978). In fact, DnaG is unable to synthesise primers on ssDNA that does not contain the highly specialised G4 ori sequence, unless DnaB is present. However, in a reaction termed general priming, DnaG, will in the presence of DnaB, synthesises primers on any naked ssDNA that can be used to prime subsequent DNA synthesis by the DNA polymerase III holoenzyme complex. Consequently, DnaB has been referred to as a "mobile promoter" of primer synthesis (Masai et al., 1990). The mechanism by which DnaG primase is activated by DnaB is unclear. However, the ATP binding function of DnaB does seem to be an essential step (Arai and Kornberg, 1981). Although physical contact between DnaB and primase is usually assumed, no direct physical or genetic evidence exists to verify this. Therefore, DnaB may act by generating a special secondary structure in the DNA template that can subsequently promote DnaG binding.

DNA polymerase III holoenzyme (DNA pol III HE).

The DNA pol III HE was originally isolated on the basis of its requirement for the replication of phage ssDNA templates in the presence of the E.coli priming apparatus (McHenry, 1982, 1991). DNA pol III HE is the largest multiprotein complex responsible for replicative DNA synthesis in E.coli. The enzyme contains 10 subunits with functions specialised for efficient replication of the E.coli chromosome. Early studies using standard fractionation procedures found that the holoenzyme actually separated into two main components: "core polymerase" and its "accessory factors". The catalytic DNA pol III core is composed of a heterotrimer of subunits that are not readily separable by chromatography. These include the 140KDa α catalytic subunit (DnaE gene product), the 23KDa ε subunit (DnaQ gene product) which is a proof-reading 3'-5' exonuclease, and the 10KDa θ subunit (DnaE gene product). Although this "core" complex has the enzymatic abilities of the holoenzyme it dissociates very quickly from the DNA template (Fay et al., 1981). The very low processive nature of the DNA pol III complex is specifically increased by the addition of the "accessory factors". These remaining subunits include: the 71KDa τ subunit, which is thought to be responsible for the dimerisation of the core DNA pol III complex (Studwell-Vaughan et al., 1991), the $\gamma\delta$ complex which is composed of γ (47KDa), δ (35KDa), δ' (33KDa), χ (15KDa) and ψ (12KDa) subunits and is involved in the ATP-dependent loading of the β subunit onto the primer terminus. The 38KDa β subunit imparts highly processive DNA synthesis to the core polymerase and is usually described as a "sliding clamp" that locks the enzyme onto the template (Stukenberg et al., 1991). Extending previous proposals that polymerases could function as dimers (Sinha et al., 1980). McHenry et al., (1991) have suggested that the

complete DNA pol III HE exists as an asymmetric dimer with distinguishable leading and lagging strand polymerases.

E.coli single-stranded DNA binding protein (E.coli SSB).

E.coli SSB is a small (monomer=18.8KDa), heat-stable protein that binds tightly to ssDNA as a tetramer (Sancar et al., 1981), forming a long chain of molecules which have a beaded appearance under the electron microscope (Chase and Williams, 1986). E.coli SSB also binds ssRNA with a 10-fold and dsDNA with a 10^3-fold lower affinity than for ssDNA. Limited proteolysis indicates that the protein consists of several domains: an N-terminal fragment of 105 amino acids in length, which binds DNA at least as well as the intact protein and therefore most likely contains the DNA binding domain and information for tetramer formation; a less structured region spanning residues 106-165, possibly involved in protein-protein interactions with other replication proteins; and a C-terminal part (amino acids 166-177) which upon deletion leads to altered kinetics of DNA binding and increases the helix-destabilising activity of the protein (Chase and Williams, 1986).

In E.coli DNA replication, the SSB is thought to be involved in assisting helicases (especially DnaB) by helping in the assembly of what is known as the E.coli primosome, (DnaB helicase/DnaG primase) onto its correct position on the DNA origin (Marians, 1984), and by melting and then stabilising regions of newly created ssDNA, lowering the possibility of strand renaturation. This melting or helix-destabilisating activity of E.coli SSB also benefits the main DNA polymerase, DNA pol III HE. in vitro polymerisation assays have demonstrated that the SSB can increase the initial rate of polymerisation and fidelity of the DNA pol III HE enzyme (Kunkel et al., 1979). The increase in polymerisation is believed to be due to the SSB's ability to remove the secondary structure

of DNA directly in front of the polymerase during replication (LaDuca et al., 1983). Alternatively, E.coli SSB could increase polymerisation by directly interacting with the accessory factors (γδ complex) in DNA pol III HE, forming a stable replication structure capable of extensive DNA synthesis. However, to date no evidence for this type of direct interaction has been found. The E.coli SSB dependent increase in fidelity is thought to be due to enhanced base-selection by the DNA polymerase, resulting from an increase in the rigidity of the DNA template upon coating by the SSB.

DNA Gyrase.

On most natural DNA templates, replication fork movement requires a topoisomerase to remove the topological strain associated with unwinding the DNA duplex. Genetic evidence and in vivo studies with topoisomerase inhibitors indicate that this function is provided in E.coli by DNA gyrase (Baker et al., 1986 ; Funnell et al., 1986). In vitro DNA replication from OriC plasmid systems depend on the addition of DNA gyrase to function properly. The gyrase is ATP-dependent and functions in replication by unlinking the parental DNA strands as they are unwound by the DNA helicase, DnaB. It is thought to achieve this by reducing the net linkage between the parental strands by two turns during each enzymatic turnover; the mechanism involves a specialised double-strand break and a DNA passing event (Wang et al., 1985).

The discontinuous nature of DNA synthesis on the lagging strand necessitates the involvement of several additional proteins; the short nascent strands require processing of primer RNA's, gap-filling and ligation to convert them to a continuous strand. RNaseH, DNA polymerase I and E.coli DNA ligase have these functions (Funnell et al., 1986 ; Ogawa et al., 1984).

11.2. Model for Elongation of E.coli DNA .

After the initiation complex has been formed at OriC (see earlier in this section), DnaB very likely starts to generate a replication fork by opening the DNA duplex, while moving 5'-3' on the strand to which it is bound. This strand would be the lagging strand; DnaG/primase, by its presumed association with DnaB, could then synthesise the multiple primers needed during lagging strand synthesis. The dimeric DNA pol III HE would most probably interact simultaneously with both template strands. The leading strand polymerase would move in the same direction as DnaB, while copying the opposite strand. To synthesise the lagging strand, the DNA polymerase would have to work on the same strand bound by DnaB, but travel in the opposite direction. The discontinuous nature of replication at the lagging strand probably necessitates the participation of the E.coli SSB to melt secondary structure in the newly exposed single-stranded template. A model of a simple replication fork is shown in figure 6 to lay out the proposed organisation of the enzymes at the fork.

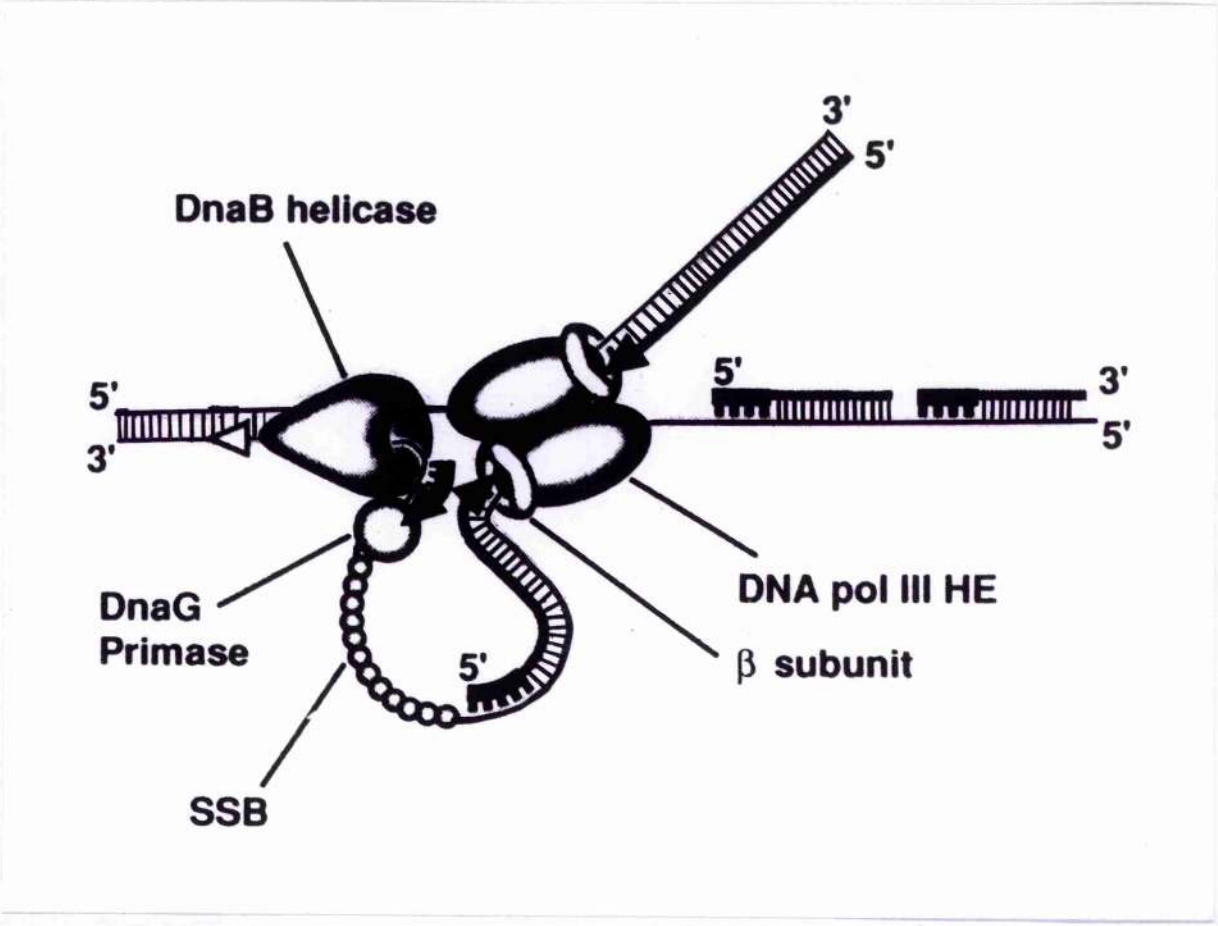


Figure 6. Model of elongation at the replication fork of E. coli (taken from Baker and Wickner, 1993).

MATERIALS and METHODS.

1. Reagents.

Reagents were obtained from BDH Chemicals Ltd, Sigma Chemical Company Ltd, and Pharmacia. All chemicals were of analar grade. Acetonitrile and HPLC grade water were obtained from Rathmans Plc. Titriated sodium borohydride was obtained from ICN. All other radiochemicals were obtained from Amersham International Plc.

2. Restriction endonucleases and DNA modifying enzymes.

Enzymes were obtained from New England Biolabs, Bishop's Stortford, Herts., England and Boehinger Mannheim Ltd., Lewes, East Sussex, England. These were used according to the manufacturers directions.

3. Bacterial culture medium.

Bacteria were propagated in Luria Broth, 10g/l bactotryptone (Difco labs, Detriot USA.) and 5g/l yeast extract (Difco) in 10mM NaCl, sterilised by autoclaving. M13 was propagated in JM101 cells grown in 2x TYE, 16g/l bacotryptone, 10g/l yeast extract, 5g/l NaCl and 1M NaOH.

4. Virus stocks.

Recombinant baculoviruses containing the Ad.5 pTP and Pol genes were obtained from E.L.Winnaker (University of Munchen). The recombinant baculovirus encoding the Ad.2 DBP gene was obtained from A.Webster (Monaghan et al., 1994) and the recombinant baculovirus encoding the NF-I DNA-binding domain was obtained from R.T.Hay (Bosher et al., 1991).

5. Plasmids.

The pEX series of plasmids containing deleted versions of the adenovirus type 2 inverted terminal repeats were as described in Hay and McDougall, (1986).

6. Oligonucleotides.

All oligonucleotides were synthesised on an Applied Biosystems 381A. The 18mer and 39mer oligonucleotides used in the strand displacement assay contained the sequences 5'-GGTATATTATTGATGATG-3' and 5'-ATTGGCTTCAATCCAAAATAAGGTATATTATTGATGATG-3' respectively, and the 42mer oligonucleotide (oligo A), which was complementary to M13mp19 DNA, had the sequence 5'-TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGG-3'. Double stranded oligonucleotides containing the binding site for NF-I (Bosher et al., 1990) and NF-κB (Clark et al, 1990) were as described. Double stranded oligonucleotides containing the binding site for NF-III contained the sequence 5'-GATCTGAGTTAATATGCAAATAAGGCGTGAG-3', (top strand).

7. Antibodies.

Monoclonal antibody α 72k B6-10 raised against Ad.2 DBP was obtained from A. Levine (Reich et al., 1983). The monoclonal IgG was purified via chromatography on a protein-G sepharose column equilibrated in PBS. Bound B6-10 antibody was eluted by extensive washing with 100mM glycine, pH 2.5 and dialysed overnight against PBS (Harlow and Lane, 1988). The anti-ova monoclonal antibody was a kind gift from A.Green and was purified in a similar fashion.

8. Preparation of single stranded φ M13 DNA.

mHR1 φ M13 DNA was obtained from R.T.Hay (Hay, 1985). Transformation of bacteria and subsequent isolation of single stranded DNA was carried out as described in Sambrook et al., (1989).

9. Quantitation of DNA.

The concentrations of all aqueous solutions of DNA were determined by scanning the absorbance between 220nm and 280nm on a Perkin-Elmer Lambda U.V/Visible Spectrophotometer. One optical density unit at 260nm was taken to correspond to 50μg/ml of double stranded DNA or 20μg/ml of single stranded oligonucleotide. Concentrations were double-checked by separating DNA samples on agarose gels containing 0.5μg/μl ethidium bromide and comparing the intensity of the fluorescence with that of known standards.

10. Preparation of Adenovirus cores.

The Adenovirus cores used in the in vitro initiation assays were prepared as Goding and Russell, (1983).

11. Radiolabelling of oligonucleotides and restriction fragments.

Single stranded oligonucleotides were labelled using T4 polynucleotide kinase (New England Biolabs). DNA was incubated in a buffer containing 50mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 5mM DTT and 0.1mM EDTA, pH 8.0 with 5 Units of T4 polynucleotide kinase and 30μCi of ($\gamma^{32}P$) ATP (Amersham, specific activity 3000 Ci/mmol) at 37°C for 45 mins. The reaction was terminated by addition of DNA dyes (20% glycerol, 50mM EDTA pH 8.0, 0.01% bromophenol blue). Labelled oligonucleotide was separated from unincorporated ($\gamma^{32}P$) ATP

and unlabelled oligonucleotide by electrophoresis on a non-denaturing polyacrylamide gel. Labelled oligonucleotide in the wet gel was identified by autoradiography with x-ray film (Fuji RX) and the gel slice containing the single stranded oligonucleotide recovered. The labelled oligonucleotide was then passively eluted from the gel slice by the addition of TEN_{100} buffer and eluted overnight at room temperature, before storage at 4°C.

Double stranded oligonucleotides and restriction fragments with overhanging 5' ends, were labelled by incubation with the Klenow fragment of E.coli DNA polymerase 1, α^{32}P dATP and α^{32}P dCTP. 50-100ng of DNA was incubated in a buffer containing 50mM Tris-HCl, (pH 7.5), 5mM $MgCl_2$, 0.1mM DTT, 100µg/ml BSA with 2 units Klenow, 100µM dNTP's (cold) and 20µCi of an appropriate (α^{32}P) radiolabelled nucleotide. The reaction was incubated at 20°C for 15 mins, then 100µM of cold nucleotide was added to chase for an additional 20 minutes at room temperature. The reaction was stopped by the addition of DNA dyes (20% glycerol, 50mM EDTA,pH 8.0 and 0.01% bromophenol blue) and the labelled DNA purified by electrophoresis as above.

The radiolabelled DNA substrate used in the exonuclease assay was internally labelled using a " T7 Quick Prime" Kit (Pharmacia LKB). The labelling reaction and subsequent purification by gel filtration were carried out as described in the manufacturers instructions.

12. Purification of Adenovirus DNA replication proteins.

12.1 Preparation of single stranded calf thymus DNA.

Type 1 calf thymus DNA (Sigma) was dissolved in water at 10mg/ml by stirring overnight and sonicating using an MSE Soniprep 150 until the

viscosity of the solution was reduced. The solution was then denatured by boiling for 15 minutes and cooled rapidly to 4°C.

12.2 Preparation of single stranded DNA-Sepharose.

100ml settled volume of Sepharose (Whatman CL-4B) was washed twice with one litre of ultra pure water, resuspended in 200ml of water and stirred gently for 30 minutes. 1.5grams of CNBr was added dropwise to the Sepharose over one minute, followed by the addition of 9ml of 10ml NaOH, dropwise over 10 minutes. The reaction was stopped by the addition of one litre of ice cold water. The slurry was then transferred to a Buchner funnel and filtered with four litres of ice cold water, followed by two litres of 10mM potassium phosphate buffer (pH 8.0). 100mg of single stranded calf thymus DNA was added and shaken gently with CNBr activated Sepharose for 12 hours at 20°C. The DNA-Sepharose was then washed with 100ml of 1M ethanolamine (pH 8.0) and gently shaken for 4 hours at 20°C in 1M ethanolamine (pH 8.0) followed by 2x200ml washes with 50mM sodium phosphate buffer (pH 8.0), 1M NaCl and finally with buffer containing 10mM Tris-HCl (pH 8.0), 1mM EDTA, pH 8.0 and 0.3M NaCl.

12.3 Preparation of hydroxylapatite resin.

1ml of powdered hydroxylapatite was added to a high salt buffer, 150mM potassium phosphate (pH 7.0), 400mM potassium chloride, 10% glycerol.The mixture was shaken and left to settle.The supernatant was discarded and a low salt buffer, 5mM potassium phosphate (pH 7.0), 400mM potassium chloride, 10% glycerol was added. This mixture was shaken and left to settle, the supernatant discarded and the slurry used in a chromatographic column.

12.4 Expression and purification of Ad.5 pTP and Pol from Spodoptera fugiperda cells infected with recombinant baculovirus.

Spodoptera frugiperda insect cells were grown in liquid culture in TC100 medium supplemented with 10% foetal calf serum at 28°C and infected with 10 p.f.u./cell of recombinant Autographa californica nuclear polyhedrosis viruses containing the genes for Ad.5 pTP and Pol. The infected cells were incubated for a further 72 hours at 28°C in TC100 supplemented in 10% foetal calf serum. The cells were harvested by centrifugation at 2000g for 10 minutes and resuspended in 25mM Hepes-KOH (pH 8.0), 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT and incubated for 20 minutes. The resuspended cells were then disrupted by 15 strokes in a Dounce homogeniser using a type B pestle, NaCl was then added to bring the final concentration to 0.2M and the extract was incubated on ice for 30 minutes. The extract was then clarified by centrifugation at 15,000g for 3 minutes and the supernatant was further clarified by centrifugation at 100,000g for 15 minutes to remove any insoluble protein.

Both Ad.5 pTP and Pol underwent the same method of purification. Clarified crude extract in 0.2M NaCl was applied to denatured calf thymus DNA-Sepharose equilibrated with 25mM HEPES-NaOH pH 8.0, 1mM EDTA, 2mM DTT, 0.2M NaCl and 105 glycerol. After extensive washing with the equilibration buffer, bound proteins were eluted with the same buffer containing 0.6M NaCl. Fractions were then collected and their concentration estimated using the Bradford's assay (Bradford, 1976). The peak fractions were pooled and dialysed overnight against two litres of 5mM KPO_4 (pH 7), 400mM KCl, 1mM DTT and 10% glycerol. The dialysate was then applied to an hydroxylapatite column equilibrated in the same buffer. After washing with the above buffer, bound protein was eluted with 50mM KPO_4 (pH 7.0), 1mM DTT, 0.4M KCl and 10% glycerol. The polypeptide composition of each fraction was monitored throughout the purification procedure

by SDS-polyacrylamide gel (8%) electrophoresis. Fractions containing pTP and pol were dispensed in small amounts and stored at -70°C. pTP-pol heterodimers were formed by mixing molar equivalents of the two proteins in storage buffer (50mM KPO_4 buffer pH 7.0, 1mM DTT, 0.4M KCl and 10% glycerol) and incubating for 20mins at 0°C.

12.5 Expression and Purification of 72KDa and 39KDa DBP from Spodoptera frugiperda cells infected with a recombinant baculovirus.

Suspension cultures (500ml) of Sf9 cells were grown to a density of $8x10^5$ cells per ml. The cells were collected by centrifugation and infected at a multiplicity of 5 p.f.u. per cell in 50ml of TC100 for 2 hours at 28°C before medium containing 5% foetal calf serum was added to 500ml and the infection was allowed to proceed for 72 hours at 28°C. Infected cells were collected by centrifugation at 2500 rpm for 15 minutes and washed in phosphate-buffered saline.

The washed cell pellet from 500ml of Sf9 cells infected with the DBP recombinant baculovirus was resuspended in 8ml of ice-cold 50mM Tris/HCl pH8, 50mM NaCl, 1mM DTT, 1mM EDTA, 1mM PMSF, 0.5% NP40 and homogenised with 20 strokes of a Dounce homogeniser using a type B pestle. The nuclei were sedimented at low speed in a microcentrifuge and the supernatant clarified by centrifugation at 60000g for 30 minutes at 4°C. The resulting cytoplasmic extract was applied to a denatured DNA-Sepharose column (10ml) equilibrated with 50mM Tris/HCl pH8, 1mM DTT, 1mM EDTA,1mM PMSF, 0.2M NaCl (buffer A). The column was washed with 3 column volumes of buffer A and eluted with buffer A containing NaCl at a concentration of 1M. Fractions containing DBP were pooled and applied (300µl aliquots), to a S-75 Superdex HR 10/30 gel filtration column (Pharmacia) equilibrated with 25mM Tris-HCl, pH 8.0, 100mM NaCl. DBP containing fractions were detected by absorbance at A_{260} nm and checked for purity on an SDS polyacrylamide gel. Purified DBP was dialysed against 25mM Tris/HCl

pH8, 100mM NaCl, 1mM DTT, 1mM EDTA, 20% glycerol, 1mM PMSF and stored at -20°C.

Purified baculovirus expressed DBP (10mg) prepared as described above was diluted to 1mg/ml in 25mM Tris/HCl pH8, 1mM DTT, 1mM EDTA, 20% glycerol and incubated with chymotrypsin (10μg/ml) for 30 minutes at room temperature. Digestion to the 39-kDa fragment was monitored by SDS-PAGE, the reaction was stopped by the addition of PMSF to 2mM and sample was dialysed against Buffer A made 0.2M with respect to NaCl. The 39-kDa DNA binding fragment was purified by single-stranded DNA-sepharose chromatography, dialysed and stored as described for the full length DBP above. Sequence analysis of the 39-kDa fragment was carried out by Bryan Dunbar at the SERC Protein Sequencing Facility (University of Aberdeen).

12.6 Expression and purification of Ad.2 72KDa DBP from adenovirus infected Hela cells.

Ad.DBP was purified from Hela cells infected at 100 p.f.u./cell for 22 hours in the presence of hydroxyurea by submitting nuclear extracts to chromatography on D.E.A.E.-cellulose and denatured DNA-sepharose, (Enomoto et al., 1981).

12.7 Expression and purification of Nuclear Factor I DNA-binding domain from Spodoptera frugiperda cells infected with a recombinant baculovirus.

The Nuclear Factor I DNA-binding domain was expressed and purified as described in Bosher et al., (1990).

12.8 Affinity purification of bacterially expressed Nuclear Factor III DNA-binding domain using the pGEX-2T bacterial fusion protein expression system.

An E.coli JM101 transformant containing the cDNA (a.a 270-582) for Nuclear Factor III DNA-binding domain (kind gift from P. O'Hare, Marie Curie Research Institute) was added to 500ml Luria Broth containing100μg/ml ampicillin and grown at 37°C until A_{600} was 0.6, IPTG was added to 0.25mM and the incubation continued for 4 hours at 25°C. Bacterial cells were then harvested by centrifugation in a Beckman JA-20 rotor at 13,000 rpm at 4°C for 3 minutes and the supernatant discarded. The bacterial pellet was resuspended in 10mls of PBS, and disrupted by sonication on ice (4x20 seconds, MSE Soniprep 150). After the addition of Triton-x-100 (final concentration of 1%) to the bacterial lysate, the lysate was clarified by centrifugation in a Beckman JA-20 rotor at 14,000rpm at 4°C for 25 minutes and the supernatant recovered and passed over a 3ml glutathione-agarose column pre-equilibrated in a PBS, 0.5M NaCl buffer. The column was then washed extensively with 15mls of PBS, 0.5mM NaCl, then eluted with 10mM reduced glutathione, 50mM Tris-HCl, pH 8.0, 0.5M NaCl and 0.25ml fractions collected. Fractions containing the fusion protein were identified by SDS polyacrylamide gel electrophoresis, pooled and the fusion protein cleaved by incubation with 8 units of human thrombin per mg of fusion protein at room temperature for 3 hours. The protease treated material was dialysed against PBS, 0.5M NaCl and reapplied to the glutathione column. Whereas the GST portion of the fusion protein bound to the column the NF-III portion failed to bind and was eluted with the above buffer.

13. Preparation of substrates to determine strand displacement activity.

DNA substrates were prepared by labelling oligonucleotides, (5pmoles) at their 5' termini with [γ^{32}P] ATP , specific activity 3000 Ci / mmol, using T4 polynucleotide kinase. Labelled oligonucleotides were isolated on an 8% non-denaturing polyacrylamide gel and by eluting overnight in 10mM Tris-HCl (pH8.0), 1mM EDTA and 100mM NaCl. DNA hybrids were formed by annealing 5 pmoles of 5'-^{32}P-labelled oligonucleotides (or restriction fragments) with 1 pmole of complementary single stranded M13 mHR1 DNA, (Hay, 1985) in 10 mM Tris-HCl (pH 8.0), 1mM EDTA and 100 mM NaCl. Following three minutes at 100^{o}C, the mixture was slowly cooled to room temperature and ^{32}P-labelled M13 DNA hybrids separated from unannealed oligonucleotide by gel filtration on a Sepharose CL-4B column equilibrated in 10 mM Tris-HCl (pH8.0), 1mM EDTA and 100mM NaCl. The 18mer-M13 DNA and 100mer-M13 DNA substrates were used in assays with Hela DBP. All assays using baculovirus DBP used the 39mer-M13 DNA substrate.

14. Preparation of substrate for determination of directionality.

8 pmoles of the 42mer oligonucleotide (oligo A) was labelled at the 5'-end with T4 polynucleotide kinase and [γ^{32}P] ATP. The labelled oligonucleotide was annealed with 2 pmoles of single-stranded circular M13mp19 DNA, by heating at 100^{o}C for three minutes and slowly cooled down to room temperature. Annealed DNA was then digested with Sma1 for three hours at 30^{o}C and the 3'-OH termini of the DNA labelled in the presence of [α^{32}P] dCTP and five units of DNA polymerase 1 (large fragment). Annealed DNA was separated from

unincorporated nucleotides by size exclusion on a 5ml Sepharose CL-4B column.

15. Preparation of linear double-stranded DNA templates.

DNA templates were prepared by digesting pEXΔ plasmids,(Hay and McDougall, 1986) with EcoR1 and BamH1. The DNA was labelled with the Klenow fragment of DNA polymerase 1. DNA fragments released were labelled at their 3' termini in the presence of [α ^{32}P] dATP, [α ^{32}P] dCTP, dGTP, dTTP (100μM) and 5 units of DNA polymerase 1 (large fragment), at 23°C for 20 mins. The dATP and dCTP concentrations were increased by the addition of unlabelled nucleotide to 100 μM and the incubation carried out for an additional 20 mins at 23°C. Labelled DNA fragments were isolated from a non-denaturing polyacrylamide gel and eluted overnight in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl.

16. Strand displacement / DNA helicase assays.

Unless otherwise indicated, reactions (30μl) were performed at 37°C for 30 mins in 40mM Tris-HCl (pH 8.0), 5mMDTT, 1mg/ml bovine serum albumin, containing 10 fmols of DNA substrate and the indicated concentrations of DBP. Reactions were terminated by the addition of 5μl of 33mM EDTA (pH 8.0), 6% sodium dodecyl sulfate, 25% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol and the products separated through an 8% non-denaturing polyacrylamide gel. Following electrophoresis, gels were dried directly onto DE81 paper (Whatman) and exposed to x-ray film, in the presence of an intensifying screen at -70°C. Radioactivity in dried gel slices was determined by liquid scintillation counting in Ecosint A. DNA helicase assays were identical to

the above except $MgCl_2$ (5mM) and ATP (1mM) were included in the standard reaction mixture.

17. Quantitation of protein.

All protein concentrations were measured using the Bradford assay (1976). 5µl of a total protein sample was mixed with 1ml of Bradford's Reagent (100mg Coomassie blue G250, 100ml orthophosphoric acid and 50ml of ethanol made up to 1 litre with distilled water), incubated at room temperature for 5 minutes and absorbance readings taken at 595nm on an LKB Biochem. Ulrtaspec II spectrophotometer. Concentrations were calculated by comparison with a standard BSA protein curve.

18.. SDS-polyacrylamide gel electrophoresis.

For protein characterisation, denaturing SDS polyacrylamide gel electrophoresis based on the method of Laemmli (1970) was carried out in a mini-gel system (Biorad Mini Protean) under reducing and non-reducing conditions.

19. Native polyacrylamide gel electrophoresis.

For gel electrophoresis DNA-binding assays and DNA unwinding assays, non-denaturing polyacrylamide gels (6% to 16%) were prepared using a 29:1 ratio of acrylamide: N,N-methylene-bis acrylamide. Gels contained 1xTBE buffer and were polymerised by the addition of 1/100 volume of 25% (w/v) ammonium persulphate and 1/2000 volume of TEMED. Gels were cast 1.5mm thick and run at 10V/cm. 1/10 volume of sample buffer (50% glycerol (v/v) 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, 100mM EDTA), was added to samples prior to electrophoresis.

20. Silver staining electrophoresis.

The Bio-Rad Silver stain was used when silver staining SDS PAGE gels and carried out according to the manufacturers instructions.

21. Agarose gel electrophoresis.

Gel slabs (100mm x 100mm) containing 1% (w/v) agrose, 1xTBE and 0.8μg/μl ethidium bromide in a total volume of 50ml, were run at 5 v/cm. One fifth volume of sample buffer (50% (v/v) glycerol, 100mMEDTA, 0.2% (w/v) bromophenol blue, 0.2%(w/v) xylene cyanol) was added to samples prior to electrophoresis.

22. Preparation of activated DNA.

Activated calf thymus DNA was prepared by the method of Fansler and Loeb, (1974). In assays containing PLP the activated DNA had Bicine substituted for Tris at the same pH and concentration.

23. Assay for DNA polymerase / residual DNA polymerase activities.

Purified adenovirus DNA polymerase or PLP inhibited polymerase were incubated with 10μg of activated calf thymus DNA, 100μM dTTP, dGTP, dCTP, 20μM dATP; 1μCi (α^{32}P) dATP (specific activity 3000 Ci/mmol), 5mM Tris, pH 8.0, 5mM $MgCl_2$ and 10mM DTT in a total volume of 50μl or 100μl for 1 hour at 37°C.Reactions were terminated by the addition of 10% TCA, 0.5% sodium pyrophosphate. Insoluble radioactivity was captured on Whatman GF/C discs by filtration under vacuum. The disc was washed twice with 10% TCA / 0.5% sodium pyrophosphate, twice with 5% TCA, once with 70% ethanol, then air-dried and the radioactivity measured by scintillation counting.

24. Assay for the modification of Ad. DNA polymerase with PLP.

Purified DNA polymerase was incubated with different concentrations of PLP at 37°C(in light resistant eppendorf tubes), in a reaction mixture containing 20mM potassium bicine, pH 8.0, 10% glycerol and 1mM EDTA. The reaction was initiated by the addition of protein. After incubation, aliquots of the reaction mix, (40µl) were withdrawn at specific time intervals and reduced with a 10-fold excess of sodium borohydride ($NaBH_4$). The reduced aliquots were allowed to stand an additional 15 minutes on ice, before being assayed for residual DNA polymerase activity. All assays were carried out in triplicate and a mean value obtained.

25. Assay for reversibility of PLP inhibition.

Purified polymerase was incubated in a reaction mixture (120µl) with 100µM PLP, 20mM potassium bicine, pH 8.0 and 10% glycerol. At timed intervals, aliquots were withdrawn and reduced with excess $NaBH_4$ and assayed for residual DNA polymerase activity. These were compared to controls treated identically, except without PLP. After 12 minutes, the reaction was split in half. To half, Tris-HCl pH 8.0 was added to 100mM, to the other half $NaBH_4$ was added to 1mM. After 20 minutes 1mM $NaBH_4$ was added to the first half and 100mM Tris-HCl, pH 8.0, was added to the second half. All assays were carried out in triplicate and a mean value obtained.

26. Substrate protection experiments.

Purified polymerase was incubated with PLP (100µM) in the presence or absence of different substrates in a volume of 50µl. The reaction buffer used was 25mM potassium bicine, pH 8.0, with various concentrations of $MgCl_2$, as indicated. After 15 minutes, a small amount of sample

(10µl) was removed and assayed for residual DNA polymerase activity. These were compared to reactions prepared identically except containing no PLP. All assays were carried out in triplicate and a mean value obtained.

Percentage protection was defined as:

$$\frac{\text{(Fraction of residual activity with substrates - fraction of residual activity without substrates)} \times 100}{\text{(1- fraction of residual activity without substrates)}}$$

27. Initial velocity analysis of PLP inhibition.

Reactions were performed using activated DNA as template-primer at different concentrations (as indicated) or with different concentrations of dNTP's (dNTP concentration was varied but the ratio of the four dNTP's was maintained at 1:1:1:1) as indicated. DNA polymerase protein was incubated in 10mM $MgCl_2$, 25mM potassium bicine, pH 8.0, in the presence of different concentrations of PLP at 37°C. Reactions were stopped at specific time intervals, by the addition of 10% TCA , 0.5% sodium pyrophosphate. All assays were carried out in triplicate and a mean value obtained.

Data was analysed by rearranging the Michaelis-Menten equation (see below) to obtain a double reciprocal plot known as a Lineweaver Burk plot :

Michaelis-Menten $$v = \frac{Vmax . (S)}{Km + (S)}$$

(Inverted equation) $$1/v = \frac{Km}{Vmax . (S)} + 1/Vmax$$

28. Glycerol gradient centrifugation.

A native (non SDS) gradient was created by layering 1ml glycerol solutions (10%, 15%, 20%, 30%) into a 5ml Beckman polycarbonate centrifuge tube. A gradient was formed by incubating the tubes at 4°C for 3 hours. A 200µl protein sample containing purified polymerase was

loaded onto the gradient and centrifuged at 49,000rpm in a Beckman SW40Ti rotor for 14 hours at 4°C. Fractions (50µl) were collected by bottom puncture and then analysed for DNA polymerase and exonuclease activities. Fractions were also run on an SDS polyacrylamide gel for identification of proteins.

29. Assay for exonuclease activity.

Nuclease activity was measured as described in Field et al., (1984).

30. Assay for the transfer of dCMP to pTP.

Purified replication proteins were incubated in a reaction mixture (10µl) containing 25mM Hepes KOH (pH 7.5), 1mM DTT, 2mM $MnCl_2$, 0.2 mg/ml BSA, 3mM ATP and 5µCi (α^{32}P dCTP), specific activity 3000Ci/mmol, with 100ng of Adenovirus cores as the DNA template (Goding and Russell, 1983) for 90 minutes at 37°C. Reactions were stopped by heating at 70°C for 5 minutes and treated with 5 units of micrococcal nuclease in the presence of 2mM $CaCl_2$ for 30 minutes at 37°C. 4µl of gel loading buffer containing 20% glycerol (v/v), 5% SDS (w/v), 570mM mercaptoethanol, 33mM Tris-HCl, pH6.7 and 0.2% bromophenol blue (w/v) was added and the samples denatured by heating at 100°C for 2 minutes. Reaction products were resolved and visualised on a 10% SDS polyacrylamide gel, which was electrophoresed, dried and subjected to autoradiography at -70°C in the presence of an intensifying screen.

Adenovirus polymerase and preterminal protein were treated with PLP and $NaBH_4$ before introduction into the **in vitro** initiation assay.

31. Radiolabelling of the adenovirus DNA polymerase active site using tritiated $NaBH_4$.

2.5 nmoles (350µg) of Adenovirus DNA polymerase was incubated with 25mM potassium bicine, pH 8.0, 0.1mM $MgCl_2$ and 0.5mM PLP in the presence and absence of 1mM dTTP and 0.1mM poly dA-oligodT_{12-18} for 15 minutes at 37°C. PLP was reduced by the addition of chilled tritiated $NaBH_4$ (specific activity= 25mCi/mmol) to a final concentration of 10mM. After all the PLP was neutralised as judged by the disappearance of yellow colour, the reaction mixture was incubated for an additional 15 minutes on ice. A small aliquot (15µl) from each reaction mixture was removed and tested for DNA polymerase activity. The reaction mix was precipitated with 10% TCA and collected by centrifugation in a Beckman TL100 at 100,000g at 4°C for 20 minutes. The precipitate was washed twice, briefly with ice cold acetone before being air drying on ice. The dried protein pellet was then redissolved in 50µl of 8M urea, 0.4M ammonium bicarbonate buffer, the urea concentration reduced to 2M by dilution with water and the polymerase protein digested with 8µg of bovine trypsin at 37°C for 2 hours. Another 8µg (1/50 w/v) of trypsin was added to the mixture and the incubation allowed to proceed overnight. After tryptic digestion peptides were analysed by reverse phase chromatography using a Waters HPLC system with a Delta-Pak C18 column. Peptides eluted from the C18 column were collected over 120 x 0.5ml fractions and 10µl aliquots from each fraction was added to 5ml of Ecoscint A and scintillation counted. Suspected peak radioactivity HPLC fractions were identified and any corresponding peptides subjected to N-terminal amino acid sequencing.

32. Buffer solutions.

The following buffers were used routinely during these studies :-

T.E.N. (10mM Tris-HCl, pH7.5, 1mM EDTA, pH8.0, 100mM NaCl).

P.B.S. (Phosphate buffered saline).

RESULTS.

Chapter 1. Purification and of Adenovirus DNA replication proteins.

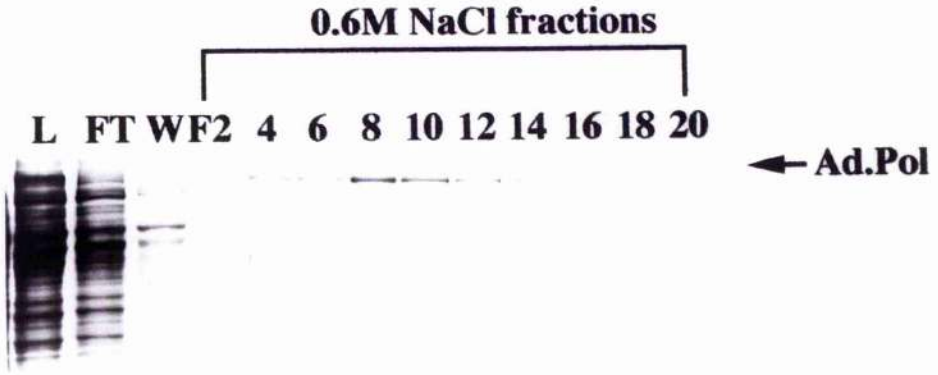
The elucidation of protein-protein, protein-DNA interactions in adenovirus infected cells (in vivo) is difficult as proteins involved in DNA replication already exist in specific protein complexes, making it difficult to determine the properties of individual proteins. The use of recombinant baculoviruses that individually express the proteins solves this problem, as well as providing large quantities of protein for characterisation.

1.1. Purification of Ad.5 polymerase from recombinant baculovirus infected Spodoptera frugiperda cells.

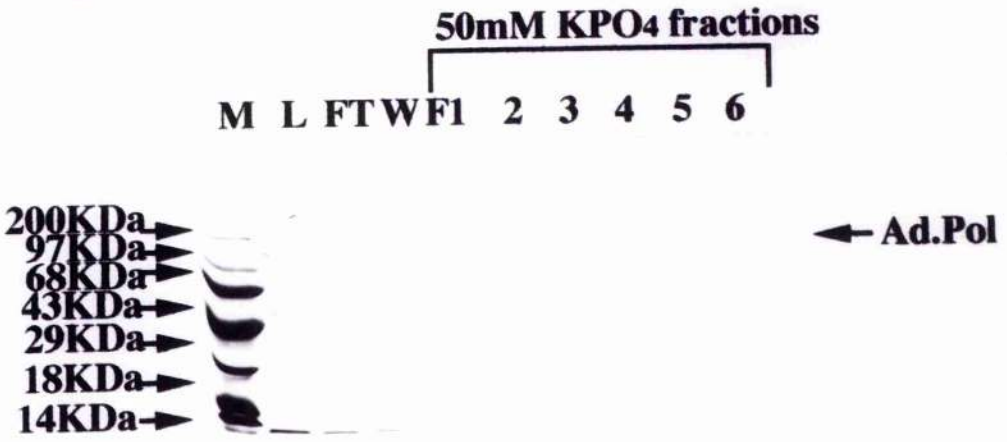
Cell extracts were prepared as described in Materials and Methods from a 500ml suspension culture of SF9 cells infected with a recombinant baculovirus containing the gene for Ad.5 DNA polymerase. Crude cell extract was adjusted to 0.2M NaCl, applied to denatured calf thymus DNA-sepharose and bound protein eluted with buffer containing 0.6M NaCl. The eluate was dialysed against 5mM KPO_4 (pH 7), 400mM KCl and 1mM DTT, applied to hydroxylapatite and bound protein eluted with buffer containing 50mM KPO_4 (pH 7). Samples of each of these fractions containing AdDNA pol were denatured and analysed by SDS-PAGE, followed by staining with Coomassie brilliant blue. A distinct species of molecular weight 140KDa corresponding to Ad.pol was observed in the crude extract (Fig 1.1A, L) and represented 70-80% of the total protein eluted from the s.s DNA-sepharose column (Fig 1.1A, F4-20). The eluate from hydroxylapatite consisted of near homogeneous pol (Fig 1.1B, F3-5). The average yield from 500mls of infected SF9 cells was 4mg of protein.

Figure 1.1 Purification of Ad.5 DNA pol from recombinant baculovirus infected insect cells. A recombinant baculovirus containing the gene for Ad.5 DNA pol was used to infect SF9 insect cells and the extract fractionated by denatured DNA affinity and hydroxylapatite chromatography. 20µl of each fraction was analysed in 10% SDS-PAGE gels. Protein species were visualised by staining with Coomassie brilliant blue R-250. The following abbreviations were used for the various tracks in:- **A.** L, crude cell extract loaded onto the s.s.DNA-sepharose column; FT, flowthrough from column; W, 0.2M NaCl buffer wash; 2-20, fractions from the s.s. DNA-sepharose column eluted with 0.6M NaCl. **B.** M, molecular weight markers; L, 0.6M NaCl s.s.DNA-sepharose fraction loaded onto the hydroxylapatite column; FT, flowthrough from column; W, 5mM KPO_4 (pH 7) buffer wash; F1-6, fractions eluted from hydroxylapatite with 50mM KPO_4 (pH 7).

A.


0.6M NaCl fractions
L FT W F2 4 6 8 10 12 14 16 18 20
Ad.Pol

B.


50mM KPO4 fractions
M L FT W F1 2 3 4 5 6
200KDa
97KDa
68KDa
43KDa
29KDa
18KDa
14KDa
Ad.Pol

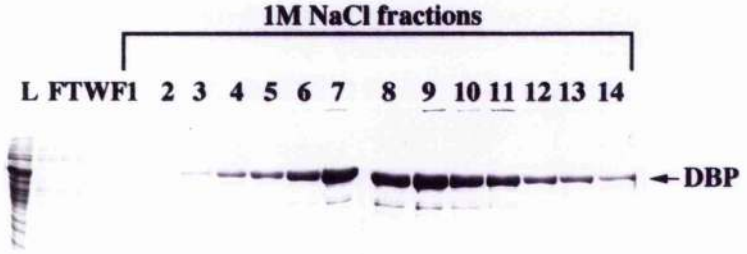
1.2. Purification of Ad.2 DBP and its 39KDa C-terminal domain from recombinant baculovirus infected Spodoptera frugiperda and full-length DBP from adenovirus infected Hela cells.

Cell extracts were prepared as described in Materials and Methods from 500mls of Spodoptera frugiperda cells infected with recombinant baculovirus expressing the gene for Ad.2 DBP (Monaghan et al., 1994). Crude cell extract was adjusted to 0.2M NaCl, applied to denatured calf thymus DNA-sepharose and bound protein eluted from the column with buffer containing 1M NaCl. Peak fractions were applied to an S-75 Superdex gel filtration column equilibrated in 25mM Tris-HCl (pH 8), 0.1M NaCl. Eluted protein was detected by absorbance at 260nm. Samples of crude cell extract, column flowthrough, wash and eluted fractions from the denatured DNA-sepharose were analysed by electrophoresis on an SDS-PAGE gel, followed by staining with Coomassie brilliant blue. A distinct species of apparent molecular weight 70KDa corresponding to DBP was observed in the crude extract (Fig 1.2A, L). The eluted fractions from the s.s. DNA-sepharose column consisted of near homogeneous DBP (Fig 1.2A, lanes 3-14). The DBPwas further purified on an S-75 Superdex gel filtration column. Eluate fractions from the S-75 Superdex column were detected by absorbance at 260nm (Fig 1.2B) and analysed by electrophoresis on an SDS-PAGE gel (Fig, 1.2 C, F7-9). Eluted fractions contained full-length DBP and a small contaminating amount of proteolysed DBP.

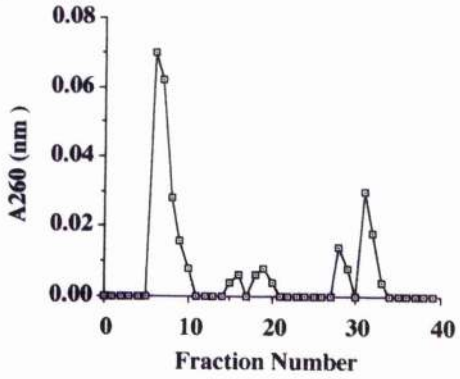
Purified baculovirus expressed full-length DBP prepared as described above was incubated with chymotrypsin (10μg/ml) for 30 mins at room temperature. After stopping the reaction with PMSF, the C-terminal proteolytic fragment was purified by single-stranded DNA-sepharose chromatography. The purified chymotryptic fragment of DBP when analysed by SDS PAGE gave a molecular weight around 39KDa.

Figure 1.2. Purification of DBP from recombinant baculovirus infected insect cells and adenovirus infected Hela cells. A. an extract of SF9 cells infected with a baculovirus containing the gene for Ad.2 DBP was fractionated by denatured DNA affinity chromatography followed by gel filtration on an S-75 Superdex column (**B** and **C**). **D.** Hela cell extracts were fractionated by D.E.A.E-Sephacel and denatured DNA chromatography. 20µl of each fraction was analysed by10% SDS-PAGE gels. Protein species were visualised by staining with Coomassie brilliant blue R-250 or silver stain in the case of Hela DBP. The following abbreviations were used for each of the tracks in :- **A.** L, crude cell extract loaded onto the s.s. DNA-Sepharose column; FT, flowthrough from column; W, wash with buffer containing 0.2M NaCl ; F1-14, fractions eluted with 1M NaCl , **C.** L, 1M NaCl s.s DNA-Sepharose fractions loaded onto the S-75 Superdex column; F6-13, fractions eluted from S-75 Superdex, detected by absorbance at 260nm, **D.** M, protein molecular weight standards; F2-18, fractions from s.s DNA-Sepharose when eluted with 1M NaCl . The positions of the molecular weight standard proteins are indicated.

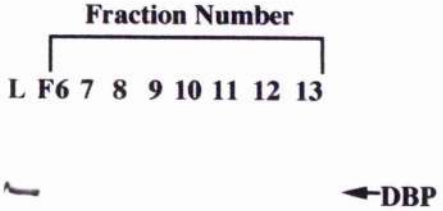
A.


1M NaCl fractions
L FTWF1 2 3 4 5 6 7 8 9 10 11 12 13 14
DBP

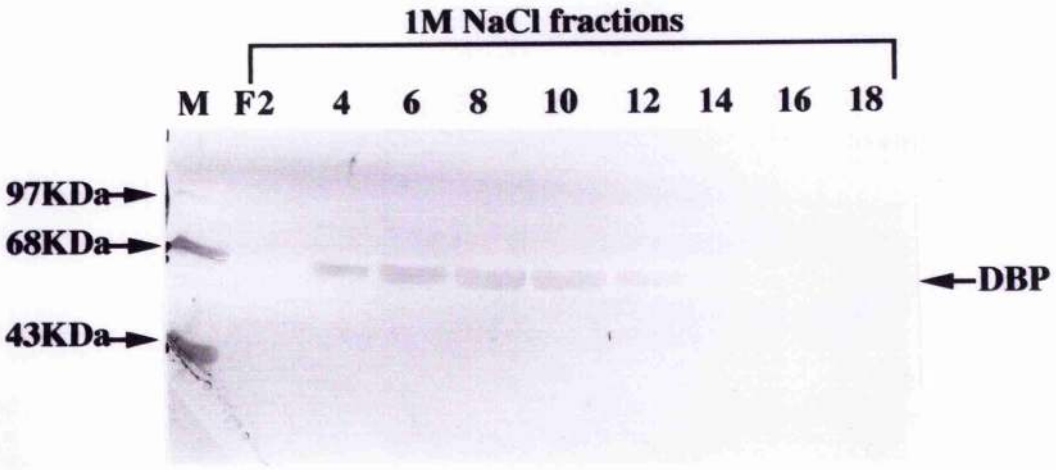
B.


0.08
0.06
0.04
0.02
0.00
A260 (nm)
0 10 20 30 40
Fraction Number

C.


Fraction Number
L F6 7 8 9 10 11 12 13
DBP

D.


1M NaCl fractions
M F2 4 6 8 10 12 14 16 18
97KDa
68KDa
43KDa
DBP

DBP was purified from adenovirus type 2 infected Hela cells as described in Materials and Methods.The crude cell extract from 10 litres of infected Hela cells was subjected to column chromatography on D.E.A.E-Sephacel and s.s. DNA-sepharose columns, respectively. Eluate fractions from the s.s.DNA-sepharose column were analysed by SDS-PAGE electrophoresis followed by silver staining and found to contain near homogeneous DBP (Fig 1.2 C, F4-12).

1.3 Purification of virally encoded Ad.5 pTP and the host encoded nuclear factor I and III DNA binding domains.

Ad.2 preterminal protein.

A recombinant baculovirus containing the gene for Ad.5 preterminal protein was expressed and purified in an identical manner to the Ad.5 DNA polymerase. The eluate from hydroxylapatite (figure 1.3, as indicated) consisted of near homogeneous pTP with a molecular weight of 80KDa.

Nuclear Factor I DNA-binding domain.

Previous studies on Adenovirus DNA replication have shown that only the purified baculovirus expressed NF-I DNA binding domain (N-terminal) was necessary for all replication functions (Bosher et al, 1991). NF-I B.D was expressed and purified as described in Materials and Methods. The purified protein was found to have a molecular weight of 35KDa when analysed by SDS PAGE (figure 1.3, as indicated).

Nuclear Factor III DNA-binding domain.

For the expression of this protein it was decided to use the E.coli / pGEX-2T system, as the bacterial pGEX-2T expression vector had

already been demonstrated to give efficient expression and easy purification of a range of eukaryotic protein species (Smith and Johnson, 1988). Furthermore, an expression vector containing the cDNA for NF-III B.D (kind gift from P O'Hare) was available for use. An E.coli culture was grown to an optical density of 0.6 (600nm) at 37°C before induction (using IPTG) of fusion protein expression. Purification of the fusion protein by glutathione agarose affinity chromatography was followed by thrombin cleavage of the fusion protein (see Materials and Methods). To purify the thrombin cleaved NF-III DNA binding domain protein from glutathione-s-transferase, the thrombin cleaved mixture was reapplied to a glutathione agarose column, washed and eluted with PBS + 0.5M NaCl. SDS PAGE analysis of the purified protein (figure 1.3, as indicated) revealed that the protein species had a molecular weight of 43,000KDa. The protein preparation also had some contaminating glutathione-s-transferase present.

Figure1.3. Purified recombinant Ad.2/5 DNA replication proteins. Adenovirus pre-terminal protein, DNA polymerase, nuclear factor I binding domain, full-length 72KDa DBP and 39KDa C-terminal DBP were all purified from the recombinant baculovirus infected insect cells, (Materials and Methods). Nuclear factor III was expressed in the pGEX bacterial fusion expression system, (Materials and Methods). Sample (5μg) were analysed on a 10% SDS-PAGE gel followed by staining with Coomassie brilliant blue R-250. The following abbreviations were used:- **Pol**, Adenovirus type 5 DNA polymerase; **pTP**, adenovirus type 5 preterminal protein; **72KDa DBP**, adenovirus type 2 DNA binding protein; **39KDa DBP**, C-terminal domain of the adenovirus type 2 DNA binding protein; **NF-I B.D.**, N-terminal domain of nuclear factor I; **NF-III B.D.**, nuclear factor III DNA-binding domain.

M
Pol
pTP
72KDaDBP
39KDaDBP
NF-I B.D.
NF-IIIB.D
M
200KDa
97KDa
68KDa
43KDa
29KDa
18KDa
14KDa

Chapter 2. Characterisation of Adenovirus DNA replication proteins.

Prompted by reports that φ 29 DNA polymerase from bacteriophage φ 29, whose genome replicates by a protein-primed mechanism similar to that of adenovirus, contained a helicase-like activity (Blanco et al., 1989) and through the previous lack of success in finding a helicase activity with Hela purified Adenovirus proteins (Field et al, 1984), the putative DNA helicase properties of recombinant baculovirus expressed adenovirus proteins, individually and in combination with each other was studied in more detail.

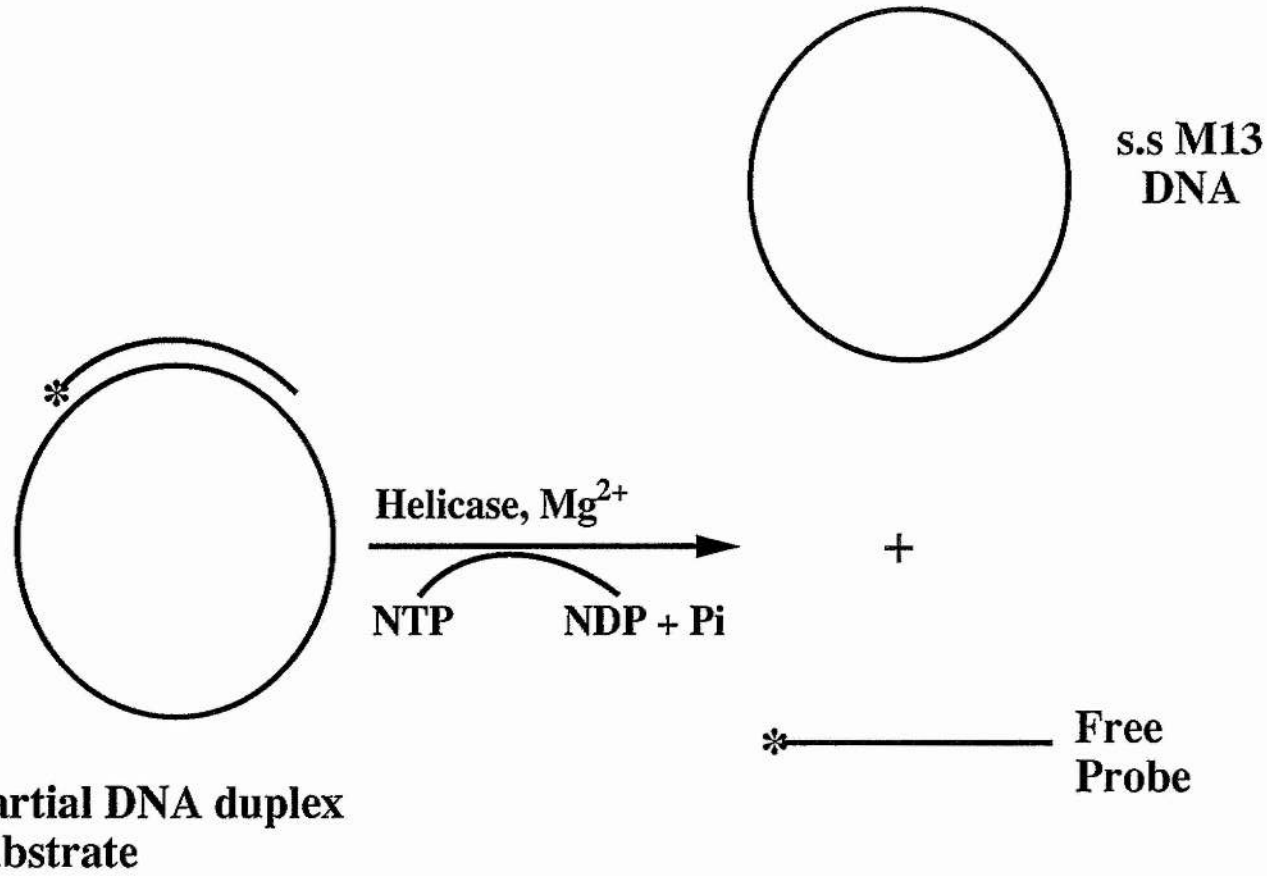
2.1 Lack of DNA helicase activity associated with purified recombinant baculovirus expressed adenovirus DNA replication proteins.

The requirement for a helicase was investigated through the use of an in vitro DNA helicase assay, originally developed by Matson et al., (1983) , as it allows reaction products to be separated and quantitated (figure 2.1). The DNA substrate used consists of a radioactively labelled complementary strand of a restriction fragment (or an oligonucleotide) annealed to a circular single stranded DNA molecule. The newly made DNA substrate is purified from unannealed labelled oligonucleotide by size exclusion on a CL-4B sepharose column (see figures 2.2A and 2.2B). The substrate is incubated with a helicase, in the presence of NTP and $MgCl_2$ and the products of the unwinding reaction resolved on a native polyacrylamide gel. The unwound labelled DNA fragment migrates faster in the gel than the substrate DNA, therefore the products of the reaction can be directly observed. The DNA substrate can also be altered in predictable ways, e.g. the length of the duplex region can be changed, the

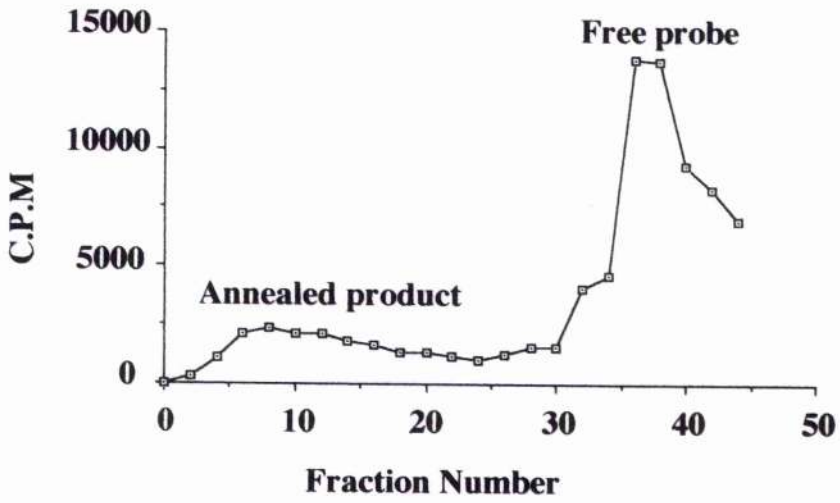
Figure 2.1. Scheme for measuring DNA helicase and DNA helix destabilising activities.

The standard DNA substrate consists of a radioactively labelled DNA strand of a restriction (or oligonucleotide) fragment annealed to its complementary sequence, a circular single stranded DNA molecule.

in vitro DNA HELICASE ASSAY.



A.



B.

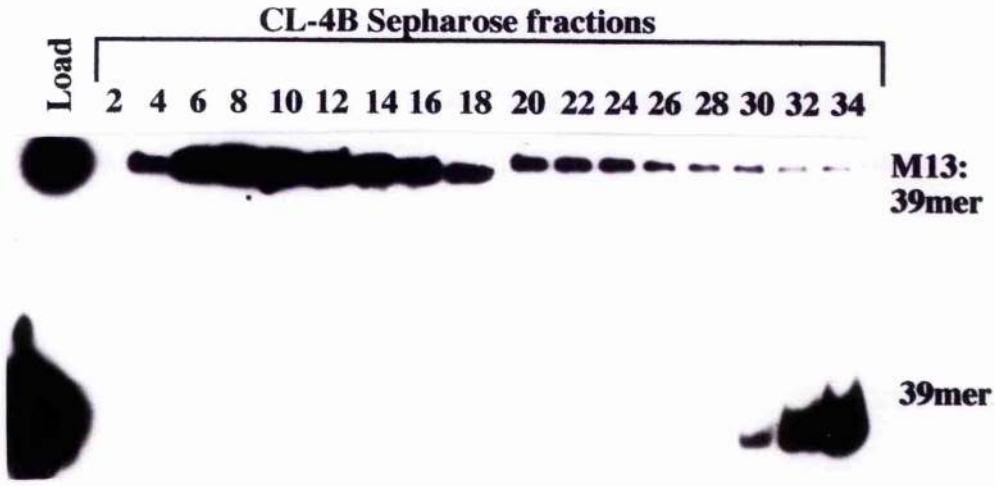


Figure 2.2. Preparation of DNA helicase / DNA helix destabilising partial duplex substrates.

Annealed and unannealed labelled oligonucleotide (or restriction fragment) were separated by gel filtration on CL-4B Sepharose. Fractions were analysed by, **A**, scintillation counting and **B**, electrophoresis on an 8% native polyacrylamide gel

substrate can be made linear, or non-homologous tails can be added onto the fragment that is to be displaced. In addition, a variation of this assay which utilises a linear molecule with labelled duplex regions at both ends, can be used to directly determine the polarity of unwinding.

The DNA substrate used in the initial studies consisted of an 18 nucleotide long oligonucleotide (18mer) annealed to a complementary single stranded M13 DNA containing the inverted terminal repeat (ITR) of the adenovirus type 2 origin of DNA replication. When annealed they formed the minimal origin of replication for Ad.2. Results from Table 2.1 show that no DNA helicase activity (using a standard partial DNA duplex substrate) was found associated with any of the recombinant proteins at the two indicated protein concentrations used.

2.2 Non-helicase / strand displacement activity of Hela Ad.2 DBP.

After initial attempts failed to show DNA helicase activity, experiments were repeated with the indicated recombinant proteins, present at concentrations used in the vitro initiation of adenovirus DNA replication assays. The possible interaction of Ad.DNA polymerase with DBP and its effect on helicase activity, if any was investigated more closely as previous studies had shown that DBP increased the processitivity of Ad.DNA polymerase (Lindenbaum et al, 1986) . Two DNA substrates were used: the 18mer previously described, and a longer partial duplex DNA substrate containing a 100 nucleotides long strand encompassing the first 100 base pairs of the Ad.2 ITR when annealed to the ss M13 DNA.

The results demonstrate, the presence of a DNA unwinding activity with both the 18mer (figure 2.3A, lanes,8-13) and 100mer (figure 2.3B, lanes,3-4, 7-8, 11-12) substrates when Ad.5 Hela DBP was present in the reaction at a concentration (17pmoles) sufficient to completely coat the

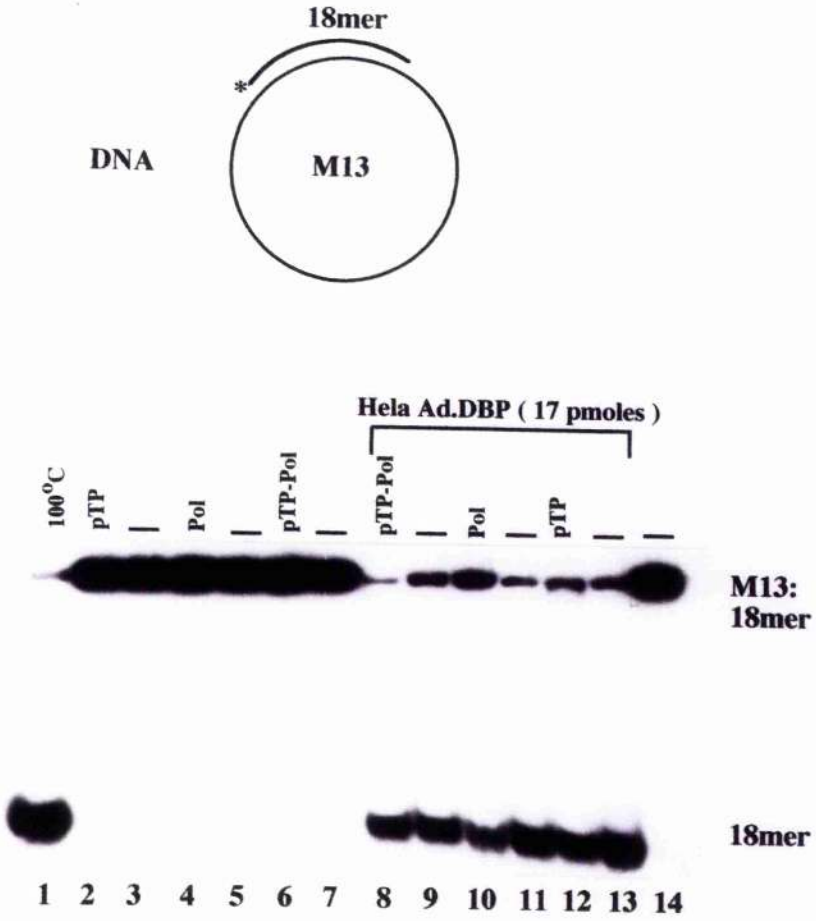
Table 2.1. Lack of DNA helicase activity associated with the Adenovirus DNA replication proteins. The DNA substrate for measuring initial helicase activity consisted of a radioactively labelled 18 nucleotide long oligonucleotide annealed to single stranded M13 DNA. After incubation with 10ng and 100ng of each protein any displaced fragment was separated from those that remained annealed to the s.s. M13 DNA by electrophoresis through an 8% native polyacrylamide gel. Helicase activity was measured as described under Materials and Methods. Complete reactions contained 40mM Tris-HCl (pH 7.5), 5mM $MgCl_2$, 5mM DTT, 1mg/ml bovine serum albumin and 10 fmoles 3'-^{32}P-labelled (18 mer) partial duplex DNA.

TABLE 2.1

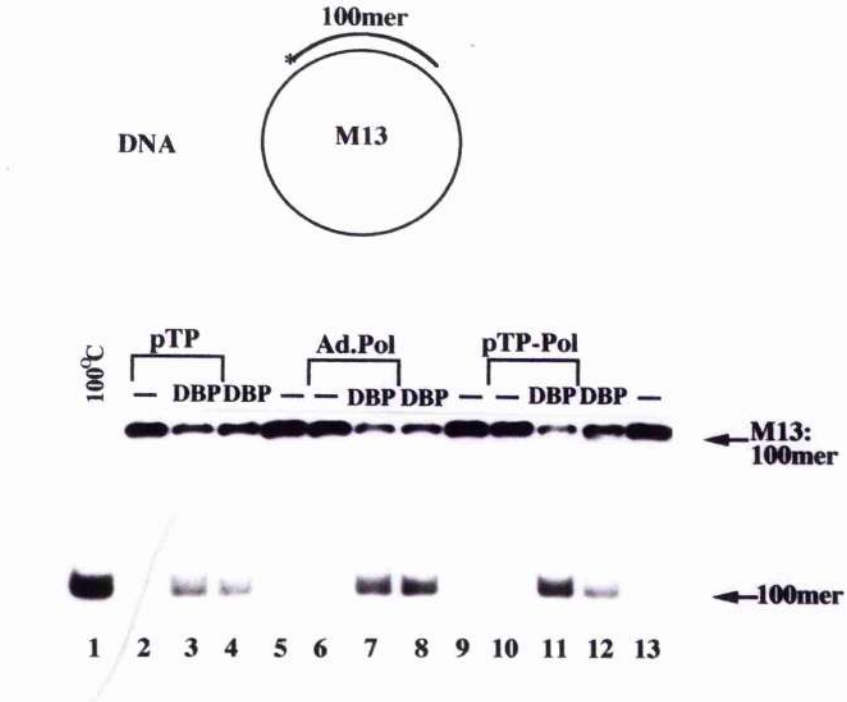
Protein species (ng) / %Displacement	NF-I B.D.		NF-III B.D.		Ad.POL		Ad. pTP		pTP-Pol Complex		Ad.DBP	
	10	100	10	100	10	100	10	100	10	100	10	100
+ dNTP (1mM)	—	—	—	—	—	—	—	—	—	—	—	—
- dNTP (1mM)	—	—	—	—	—	—	—	—	—	—	—	—
+Mg (5mM)	—	—	—	—	—	—	—	—	—	—	—	—
+EDTA (50mM)	—	—	—	—	—	—	—	—	—	—	—	—
+ NaCl (25,50,100 mM)	—	—	—	—	—	—	—	—	—	—	—	—
+rNTP (1mM)	—	—	—	—	—	—	—	—	—	—	—	—
-rNTP (1mM)	—	—	—	—	—	—	—	—	—	—	—	—

Figure 2.3. Hela DNA binding protein exhibits a non helicase DNA unwinding activity. Two DNA substrates for measuring the helicase activity of Hela DBP were constructed as in Materials and Methods. One consisted of an 18 nucleotide long oligonucleotide (18mer) annealed to M13 single-stranded DNA as described above, the other was a 100 nucleotide long single strand (from a restriction fragment) annealed to single stranded M13 DNA; **A.** native gel analysis of the reaction products after incubation of the 18mer substrate (10 fmoles) with the adenovirus replication proteins, in the presence and absence of saturating amounts (17pmoles) of Ad.5 Hela DBP. Lane 1, heat denatured control; lanes, 3,5,7 and14, no protein; lanes, 2,4 and 6, 10ng of preterminal protein (pTP), polymerase (Pol) and the preterminal-polymerase heterodimer complex (pTP-Pol), respectively, in the absence of DBP; lanes 8,10 and 12, contain 10ng of pTP, Pol and pTP-Pol respectively in the presence of DBP; lanes 9, 11 and 13, contain DBP only, at saturating levels (17pmoles).**B**. native PAGE analysis of the 100mer DNA helicase / DNA helix destabilising reaction products, in the presence and absence of 17pmoles DBP; Lane 1, heat denatured control; lanes, 5, 9 and 13, no protein; lanes 2, 6 and 10, 10ng of pTP, pol and pTP-pol, respectively, in the absence of DBP; lanes, 3, 7 and 11, contain 10ng pTP, pol and pTP-pol in the presence of DBP (17pmoles); lanes 5, 9 and 12, contain DBP only at saturating levels (17pmoles).

A.


18mer
*
DNA
M13
Hela Ad.DBP (17 pmoles)
100°C
pTP
Pol
pTP-Pol
pTP-Pol
Pol
pTP
M13:
18mer
18mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14

B.


100mer
*
DNA
M13
100°C
pTP
Ad.Pol
pTP-Pol
DBP DBP
DBP DBP
DBP DBP
M13:
100mer
100mer
1 2 3 4 5 6 7 8 9 10 11 12 13

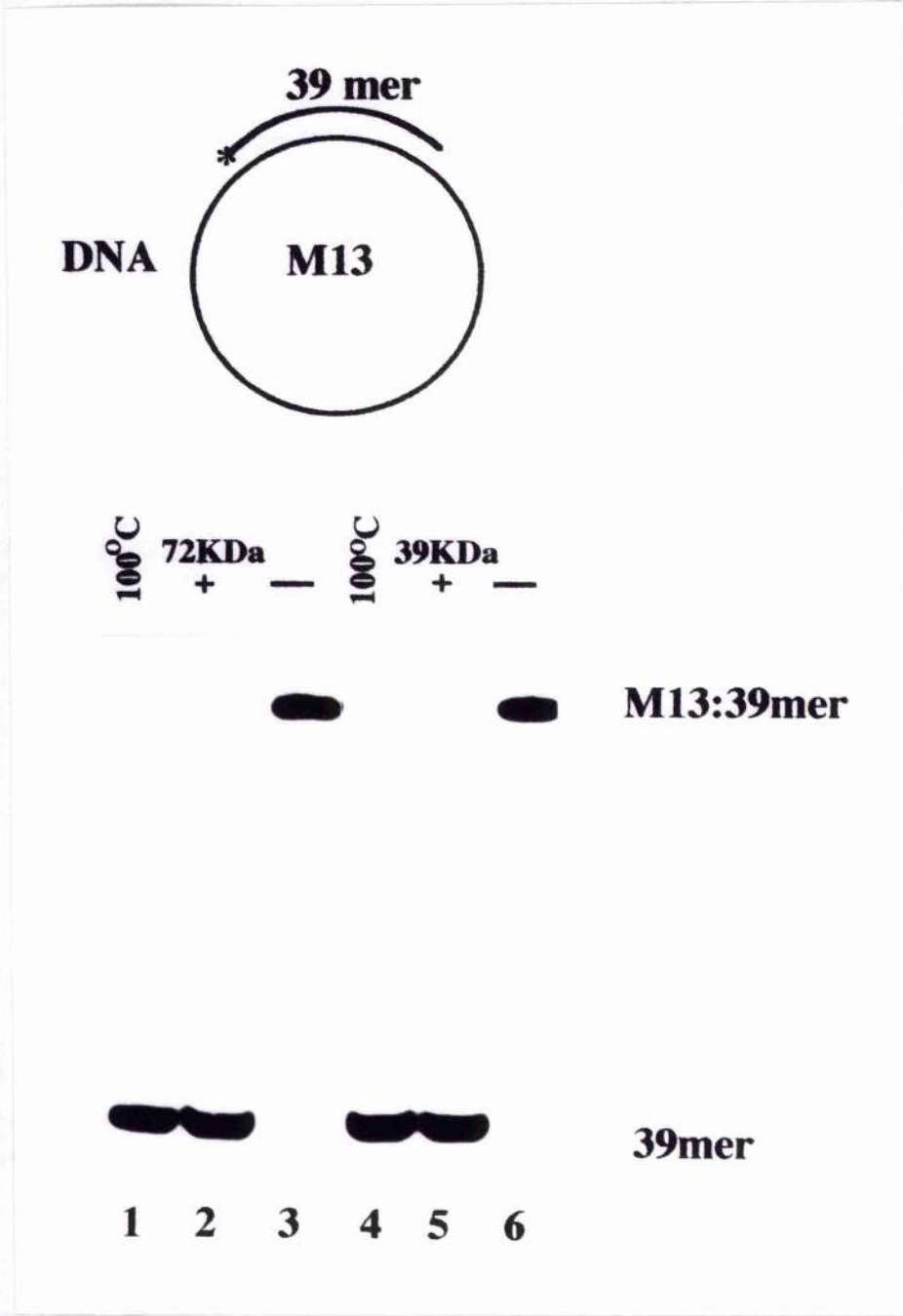
single stranded region of the DNA substrate. With the 18mer, DBP unwound approximately 80-90% of the annealed DNA fragment with this figure falling to 50-60% with the larger template. The other protein species pTP, Pol, and the heterodimer pTP-Pol showed no activity with the 18mer (figure 2.3A, lanes, 2, 4 and 6, respectively) or 100mer template (figure 2.3B, lanes, 2, 6 and 10, respectively).

2.3 Helix destabilisation activity of the recombinant baculovirus Ad.2 DBP and 39KDa C-terminal DBP .

Although adenovirus type 2 DNA binding protein (DBP) can be purified in high yield from adenovirus infected HeLa cells it is not straightforward to genetically manipulate the DBP in this genetic background. The gene for DBP was therefore inserted into a modified baculovirus genome which was used to isolate recombinant baculovirus that would express DBP in infected insect cells. To further characterise the biochemical properties of DBP that might be involved in viral DNA replication, both baculovirus expressed full-length 72KDa DBP and the 39KDa chymotryptic fragment of DBP were tested for their ability to unwind short stretches of double stranded DNA, as was the case for DBP isolated from adenovirus type 2 infected Hela cells.The substrate utilised to assay for this activity was a ^{32}P labelled 39mer oligonucleotide annealed to the single stranded form of M13 DNA.This substrate was incubated with concentrations of both 72KDa and 39KDa DBP that were sufficient to completely coat the M13 single stranded DNA and the ^{32}P labelled DNA products of the reaction analysed on a native polyacrylamide gel after removal of bound protein by denaturation in the presence of SDS. Both the 72KDa and 39KDa DBP proteins were capable of releasing the 39 mer oligonucleotide from the M13 DNA substrate, resulting in the appearance of free oligonucleotide (lanes 2 and 5, figure 2.4). Incubation

Figure 2.4. DBP and 39KDa C-terminal DBP isolated from recombinant baculovirus infected insect cells exhibit a non helicase strand displacement activity.

The substrate used was a ^{32}P-labelled 39 base-pair oligonucleotide annealed to single stranded M13 DNA as described in Materials and Methods. The substrate, shown in the upper part of the figure was incubated with 72KDa and 39KDa DBP, present at a concentration sufficient to completely saturate the 39mer-M13 DNA substrate. Reaction products were analysed on a native polyacrylamide gel. Lanes 1 and 4, heat denatured controls; Lanes 2 and 5, contain 34pmoles of 72KDa and 39KDa DBP, respectively; lanes, 3 and 6, no protein.


39 mer
*
DNA
M13
100°C
72KDa
+
—
100°C
39KDa
+
—
M13:39mer
39mer
1 2 3 4 5 6

of the substrate in the absence of DBP did not result in the release of labelled 39 mer oligonucleotide (lanes 3 and 6, figure 2.4) indicating that the DNA duplex was stable under the conditions employed for the reactions.

2.4 Characterisation of the strand displacement activity of recombinant baculovirus DBP.

One of the surprising properties of the DNA unwinding activity described above, and with Hela DBP, was that it appeared to take place in the absence of any added energy source. To investigate this further, reactions were incubated in the presence of a variety of nucleotide triphosphates or their analogues and DBP catalysed strand displacement quantitated after electrophoretic separation of the products. Strand displacement activity was not stimulated by the addition of ATP, CTP, UTP, dATP, dCTP or the presence of an ATP regeneration system and activity was not inhibited by the addition of the ATP analogue ATPγS (Table 2.2). Similarly, addition of EDTA or $MgCl_2$ to 2mM was without consequence (Table 2.2). These data confirm that the strand displacement activity of DBP is independent of an energy source and does not require the presence of a divalent cation. In fact, addition of $MgCl_2$ has a strong inhibitory effect on the strand displacement activity with 80% inhibition at 8mM $MgCl_2$ (figure 2.5C). Inclusion of NaCl in the reaction also reduces strand displacement activity of DBP with less than 30% of the activity remaining at 100mM NaCl (figure 2.5A). Titration of increasing amounts of both the 72KDa and 39KDa proteins into the reaction revealed that strand displacement only took place after the DNA was completely covered with bound protein (figure 2.5D). At saturating DBP concentrations the unwinding reaction is rapid with all of the 39 mer

Table 2.2. Requirements for baculovirus derived 72KDa DBP strand displacement activity with 39mer-M13 DNA partial duplex substrate. The displacement activity was measured as described in Materials and Methods, with the indicated additions, in the presence of 34pmoles of 72KDa DBP. Complete reactions contained 40mM Tris-HCl (pH 8), 5mM DTT, 1mg/ml bovine serum albumin, 34 pmoles 72KDa DBP and 10 fmoles of 3'-^{32}P-labelled partial duplex DNA.

TABLE 2.2

Additions	Concentration mM	% Displacement
COMPLETE	—	82
COMPLETE + $MgCl_2$	2	81
COMPLETE + EDTA	50	83
COMPLETE + ATP	1.5	79
COMPLETE + ATP Regeneration System	—	81
COMPLETE + $ATP_\gamma S$	1.5	78
COMPLETE + $ATP_\gamma S$ and ATP	1.5	79
COMPLETE + CTP	1.5	78
COMPLETE + UTP	1.5	77
COMPLETE + dATP	1.5	78
COMPLETE + dCTP	1.5	80

oligonucleotide released from the M13 DNA within 1 minute (figure 2.5B).

2.5 Involvement of the C-terminal region of Ad.DBP in strand displacement.

To identify which region of the protein was involved in the strand displacement activity and to confirm that strand displacement was being catalysed by DBP rather than a copurifying contaminating protein, the DBP specific B6-10 monoclonal antibody (Reich et al, 1983) purified to homogeneity from a protein G-Sepharose column was included in the unwinding reaction. Eluted fractions (figure 2.6A, F1-5) were incubated overnight against PBS before addition to the unwinding reaction. While inclusion of purified IgG from a monoclonal antibody raised against ovalbumin had no effect on the unwinding activity of DBP, equivalent amounts of antibody specific for DBP strongly inhibited strand displacement activity (figure 2.6B). The epitope recognised by the B6-10 antibody is present within the C-terminal chymotryptic fragment of DBP (Reich et al, 1983) and thus confirms that this region of the protein, which retains DNA binding activity, participates in the unwinding activity of DBP.

2.6. Directionality of strand displacement reaction.

The circular nature of the partial duplex routinely used as a helicase substrate (figure 2.1) does not allow the assignment of directionality to the DNA unwinding activity. A linear DNA substrate with short duplex regions at either end and a large internal single stranded region was therefore constructed (figure 2.6C upper level). The duplex regions of the substrate contain ^{32}P labelled DNA of 28 and 15 nucleotides such that if DBP bound to the single stranded region and migrated on the DNA in a 5'

Figure 2.5. Effect of ionic strength, protein concentration and time on the strand displacement activity. Using the standard assay as described under Materials and Methods, 34pmoles 72KDa DBP was incubated with varying concentrations of, **A.** NaCl and **C.** $MgCl_2$. **B.** Protein concentrations of 72KDa and 39KDa DBP ranging from 0-80 pmoles were incubated with the substrate as above. **D.** the time dependence of strand displacement was measured in a reaction mixture (90µl) containing 200 pmoles of 72KDa DBP. Aliquots (10µl) were withdrawn at the indicated times and loaded directly onto a native polyacrylamide gel for analysis.

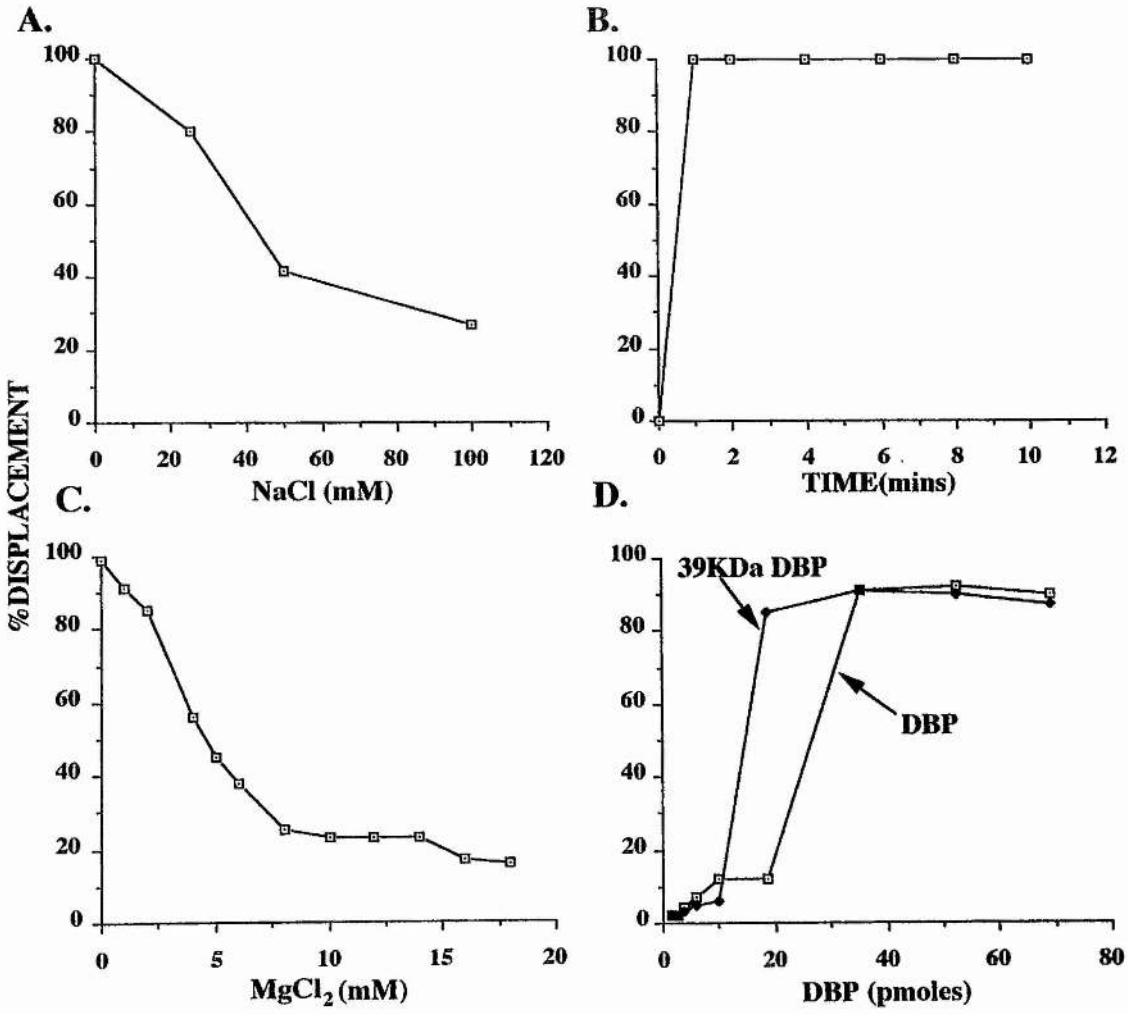
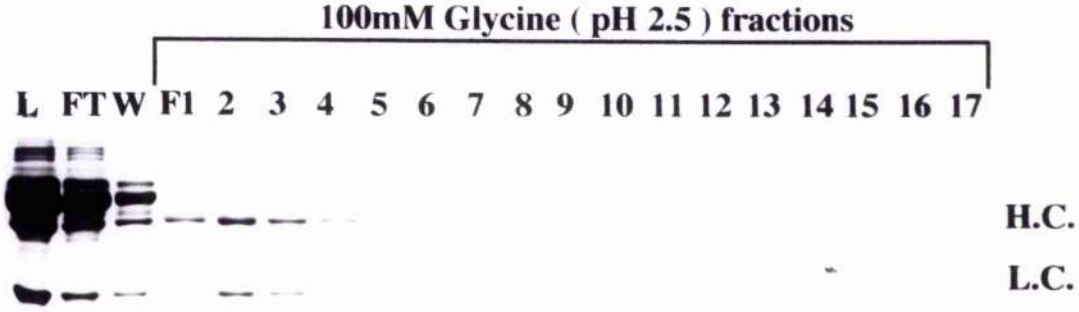
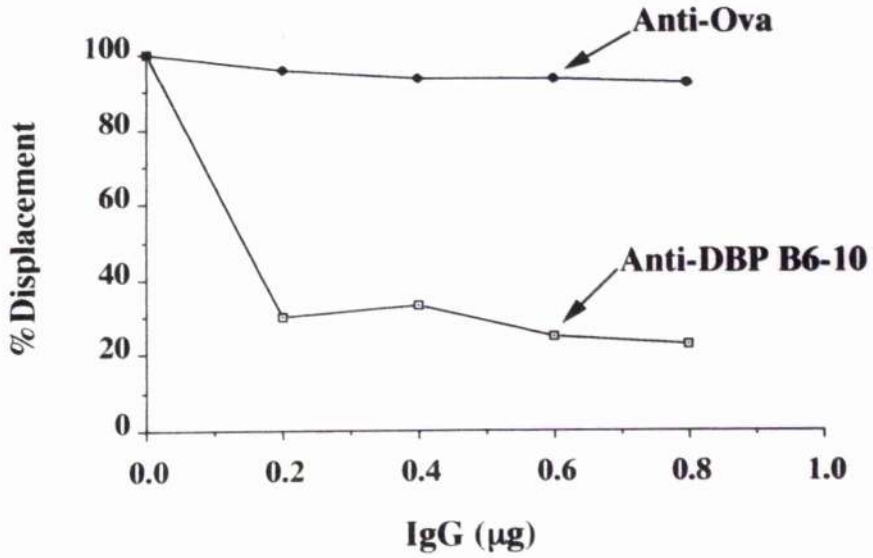

A.
100
80
60
40
20
0
0
20
40
60
80
100
120
NaCl (mM)
%DISPLACEMENT
B.
100
80
60
40
20
0
0
2
4
6
8
10
12
TIME(mins)
C.
100
80
60
40
20
0
0
5
10
15
20
MgCl2 (mM)
D.
100
80
60
40
20
0
0
20
40
60
80
39KDa DBP
DBP
DBP (pmoles)

Figure 2.6. The 39KDa C-terminal subunit of Adenovirus DNA binding protein is responsible for strand displacement activity which occurs with no directionality. A. Mouse ascitic fluid B6-10, containing monoclonal antibodies against the C-terminal of Ad.2 DBP, was purified on protein G-Sepharose. Bound antibody was eluted with 100mM glycine, pH 2.5, collected in 1/20 volume 1M sodium phosphate, pH 8 and analysed on an 8% SDS-PAGE gel. 20µl of each fraction was analysed by SDS PAGE. Heavy chain (H.C.) and light (L.C.) were visualised by staining with Coomassie brilliant blue R-250. The following abbreviations were used for the various tracks:- L, ascitic fluid from mouse; FT, flowthrough from column; W, PBS pre-elution wash; F1-17, fractions eluted with 100mM glycine pH2.5 . **B.** 17 pmoles of 72KDa DBP were incubated with increasing amounts of anti-DBP (anti-DBP B6-10) and anti-Ovalbumin monoclonal antibodies (anti-Ova). After 30 mins at 0°C the fractions were measured for DNA unwinding activity as described in Materials and Methods. **C.** 39KDa DBP (51-3.25 pmoles) was incubated with a DNA substrate used to measure the direction of unwinding (as indicated at the top of the panel, prepared under Materials and Methods).Lane 1, heat denatured control; lanes 2-6, substrate incubated with the indicated amounts of 39KDa DBP; lane 7, no protein.

A.

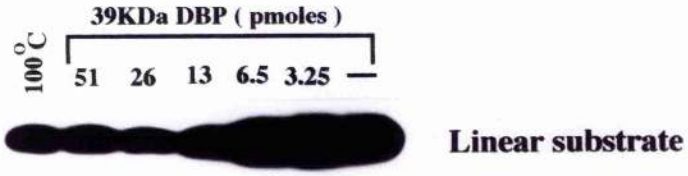

100mM Glycine (pH 2.5) fractions
L FT W F1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
H.C.
L.C.

B.


Anti-Ova
Anti-DBP B6-10
% Displacement
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
IgG (μg)

C.

Fragment Displaced	Direction of Movement
15mer	3' to 5'
28mer	5' to 3'

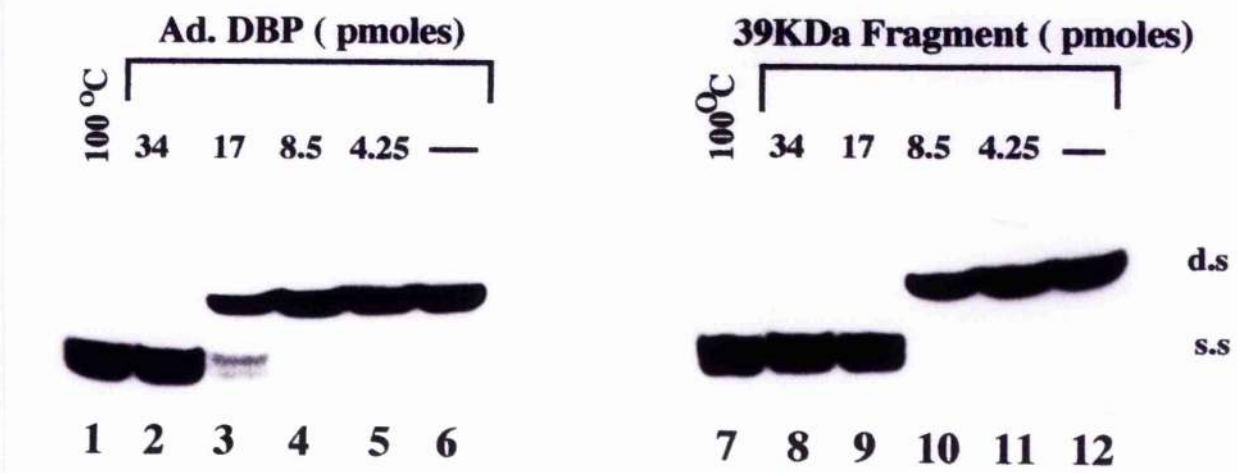


to 3' direction then the 28 nucleotide fragment would be displaced, whereas if DBP migrated in a 3' to 5' direction on the single stranded DNA then the 15 nucleotide fragment would be released (figure 2.6C). In fact incubation of this substrate with the 39KDa DBP resulted in both fragments being displaced by DBP although the smaller fragment, being more easily unwound, was detected at a lower concentration of 39KDa DBP. Thus it appears that unlike ATP dependent helicases the strand displacement activity of adenovirus DBP is not strictly directional.

2.7. Ad.DBP promoted melting of linear duplex DNA.

In the assays described above the substrate used is largely single stranded with only a short stretch of double stranded DNA. Although DBP binds cooperatively and tightly to single stranded DNA it has also been shown that DBP can bind to double stranded DNA (Cleat & Hay, 1989; Stuiver et al., 1990). It was therefore of interest to determine if DBP could unwind a completely double stranded DNA fragment. A ^{32}P labelled 27 b.p double-stranded DNA fragment which contains base pairs 1-18 of the adenovirus type 2 origin of replication was incubated with both DBP and the 39KDa proteolytic fragment and the products separated on a native polyacrylamide gel which is capable of resolving single and double stranded DNA (figure 2.7). Both DBP and the 39KDa proteolytic fragment of DBP were capable of unwinding the short DNA duplex, although again it was noticed that the 39KDa fragment appeared to be more active in the assay than DBP with maximal activity being achieved at a twofold lower concentration of protein. Under the conditions of the assay the duplex was stable in the absence of DBP (figure 2.7, lanes 6 and 12). To determine the size of double stranded DNA that could be unwound by DBP, a series of ^{32}P labelled double stranded DNA fragments of increasing size were prepared from deleted versions of the

Figure 2.7. Ad.DBP promoted melting of linear duplex DNA.
Reactions were performed using the standard displacement assay. The indicated amounts of 39KDa and 72KDa DBP (34-0 pmoles) were incubated with a short ^{32}P-labelled 27 base-pair linear DNA molecule containing the first 18 base-pairs of the adenovirus type 2 origin of DNA replication. Duplex and single stranded DNA were separated by electrophoresis through a 16% native polyacrylamide gel. Positions of the native (d.s) and denatured (s.s) DNA's are shown. Lanes 1 and 7, heat denatured controls; lanes 2-6, 72KDa DBP (34-0 pmoles); lanes 8-12, 39KDa DBP (34-0 pmoles).

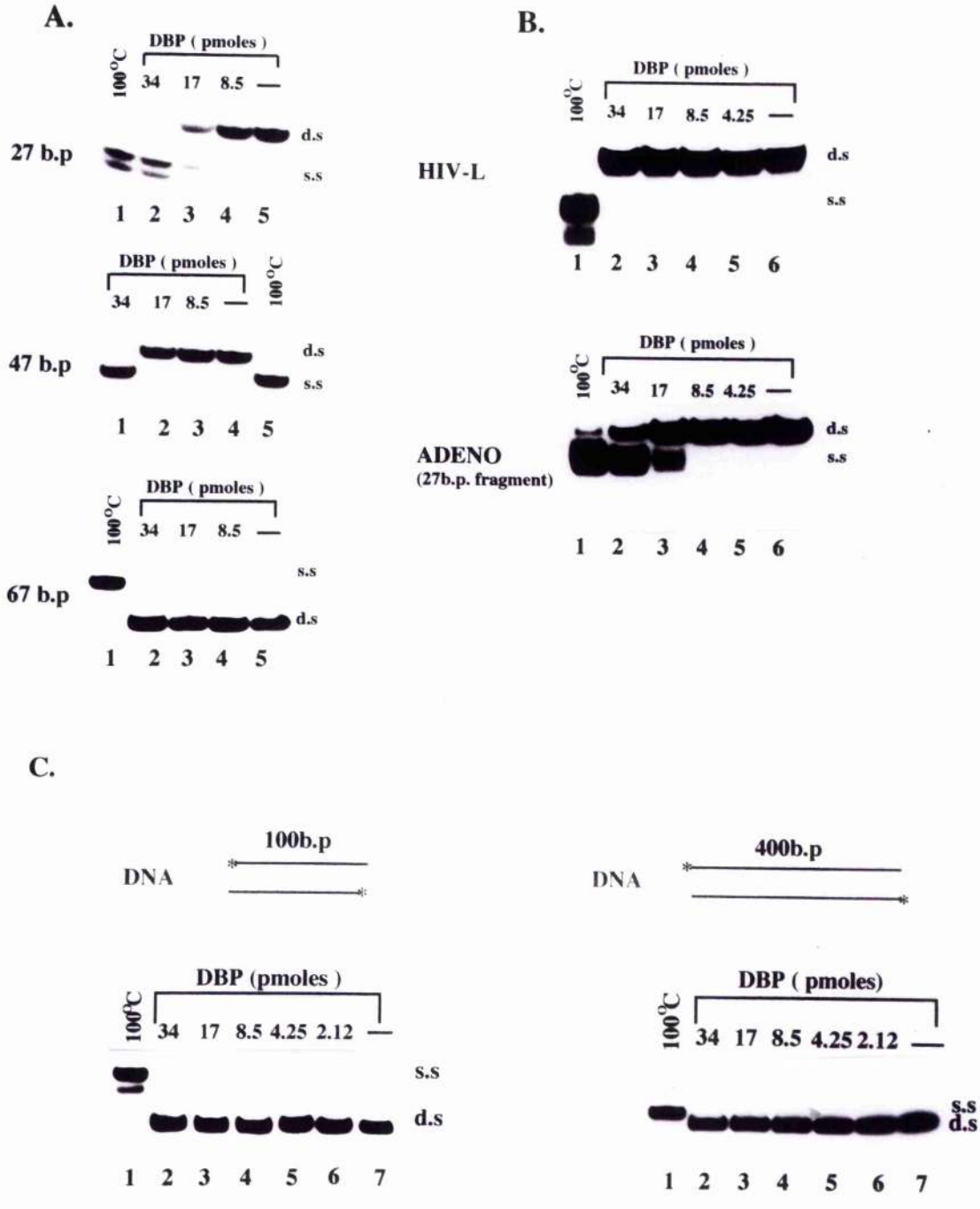

Ad. DBP (pmoles)
100 °C
34
17
8.5
4.25
—
1 2 3 4 5 6
39KDa Fragment (pmoles)
100°C
34
17
8.5
4.25
—
d.s
s.s
7 8 9 10 11 12

adenovirus type 2 origin of DNA replication (Hay and McDougall, 1986). Three linear templates, figure 2.8A, containing 27 (1-18 Ad2 origin), 45 (1-36 Ad2 origin) and 67 (1-58 Ad2 origin) base-pairs of double-stranded DNA were incubated with DBP and the products of the reactions fractionated on native polyacrylamide gels. DBP unwound the 27 and 45 base-pair templates, but not the 67 base-pair substrate. Larger DNA substrates of 100 b.p and 400 b.p. could not be unwound by Ad.DBP even at very high protein concentrations, (figure 2.8C). To determine if the observed strand displacement activity on completely double stranded DNA fragments was dependent on base composition of the DNA Ad DBP was incubated with two linear, duplex templates of similar size but different base composition. One DNA fragment of 23 base pairs representing the binding site for the transcription factor NF-κB (HIV-L) and had a G+C content of 58% while the other fragment of 27 base pairs contained the first 18 base pairs of the adenovirus type 2 origin (ADENO) and had a G+C content of 30%, were used as DNA substrates. While Ad. DBP could separate the strands of the DNA duplex with low G+C content (ADENO, figure 2.8B) it was unable to unwind the strands of the DNA duplex with high G+C content (HIV-L , figure 2.8B). DBP therefore appears to be unable to unwind completely double stranded DNA fragments of high structural stability.

2.8. Strand displacement of DNA duplexes by DBP is inhibited by bound proteins.

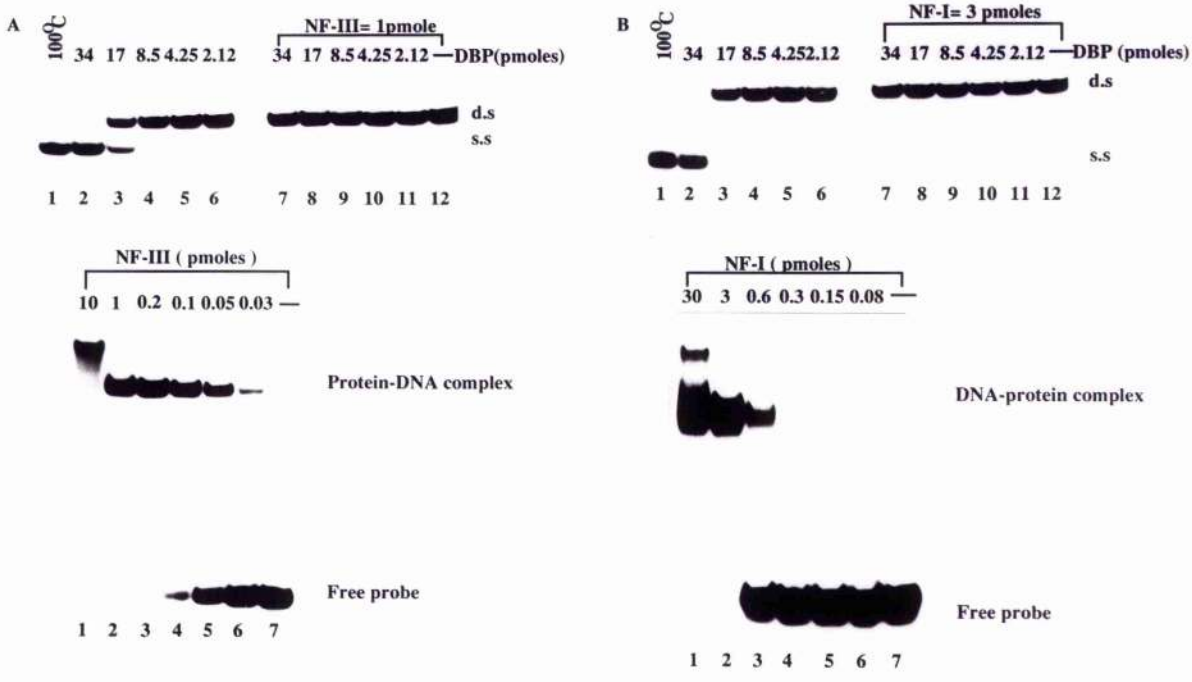
Prior to initiation of adenovirus DNA replication, the origin is bound by the sequence specific DNA binding proteins NFI and NFIII. It was thus of interest to determine if DBP could unwind DNA duplexes which were already bound by these proteins. DBP was therefore incubated with double stranded oligonucleotides containing the binding sites for NFI and

Figure 2.8 Ad.DBP linear duplex DNA strand displacement is dependent on size and thermal stability. Reactions were performed as described under Materials and Methods. **A.** Varying amounts of 72KDa DBP (34-0 pmoles) were incubated with a series of ^{32}P-labelled double stranded DNA fragments of different size. The DNA templates used in A were 27 base pairs long containing base pairs 1-18 of the Ad.2 origin, 47 base pairs long containing base pairs 1-36 of the Ad.2 origin and 67 base pairs long containing base pairs 1-58 of the Ad.2 origin, respectively. Positions of the native (d.s) and denatured (s.s) DNA's are shown. 27 base pairs ; lane 1, heat denatured control; lanes 2-5, 34-0 pmoles DBP; 45 base pairs; lane 5, heat denatured control; lanes 1-4, 34-0 pmoles DBP; 67 base pairs; lane 1, heat denatured control; lanes 2-5, 34-0 pmoles DBP. **B.** Varying concentrations of 72KDa DBP (34-0 pmoles) were incubated with two ^{32}P-labelled DNA fragments containing the binding site for the transcription factor HIV-L NF-κB (23 base pair template) and the adenovirus type 2 origin of replication (27 base pair template); **HIV-L**; lane 1, heat denatured; lanes 2-6, 34-0 pmoles DBP; **ADENO**; lane 1, heat denatured control; lanes 2-6, 34-0 pmoles DBP. **C.** Reactions were carried out as in A, with larger linear substrates that contained the first 100 base pairs and 400 base pairs of the adenovirus type 2 genome. The lane set-up was the same for both templates; lane 1, heat denatured control, lanes 2-7, 34-0 pmoles DBP.


A.
DBP (pmoles)
100°C
34 17 8.5
27 b.p
d.s
s.s
1 2 3 4 5
47 b.p
67 b.p
B.
HIV-L
34 17 8.5 4.25
1 2 3 4 5 6
ADENO
(27b.p. fragment)
C.
DNA
100b.p
400b.p
DBP (pmoles)
34 17 8.5 4.25 2.12
1 2 3 4 5 6 7

NFIII either in the absence or the presence of the cognate bound protein. To ensure that the specific binding sites were fully occupied by either NFI or NFIII, increasing quantities of the two proteins were incubated with the ^{32}P-labelled double stranded oligonucleotide and the formation of DNA-protein complexes determined in a gel electrophoresis DNA binding assay. Amounts of NFI (1 pmole) and NFIII (3 pmoles) were therefore chosen that resulted in full site occupancy but higher order complexes, indicative of non-specific binding, were not detected (figure 2.9, A and B lower panels). DBP was incubated either with the double stranded oligonucleotides or with the double stranded oligonucleotides already bound to NFI or NFIII. While DBP can unwind the DNA duplexes it is unable to do so when either NFI or NFIII are bound to their cognate sites (figure 2.9 A and B, upper panels). Therefore, it seems unlikely that DBP alone could be responsible for helix opening at the origin prior to initiation of DNA replication.

Figure 2.9. Bound NF-I and NF-III inhibit 72KDa DBP promoted DNA unwinding. Reactions were performed as described under Materials and Methods. **A.** Upper panel; various concentrations of 72KDa DBP (34-2.12 pmoles) added into the standard reaction containing an NF-I binding site template , in the presence and absence of saturating amounts of Nuclear Factor I (1 pmole). Lower panel; gel electrophoresis DNA binding assay as described under Materials and Methods, with the 33mer double-stranded oligonucleotide containing the binding site for NF-1. Lanes 1-7, 10-0 pmoles NF-I. Positions of the DNA-protein complexes and free probe are indicated. **B.** Upper panel; various concentrations of 72KDa DBP (34-2.12 pmoles) added into the standard reaction containing an NF-III binding site template, in the presence and absence of saturating amounts of Nuclear Factor III (3 pmoles). Lower panel; gel electrophoresis DNA binding assay, containing the 30mer double-stranded oligonucleotide containing the binding site for NF-III. Lanes 1-7, 30-0 pmoles NF-III. Positions of DNA-protein complexes and free probe are indicated.


A
100℃
34 17 8.5 4.25 2.12
NF-III= 1pmole
34 17 8.5 4.25 2.12 —DBP(pmoles)
d.s
s.s
1 2 3 4 5 6 7 8 9 10 11 12
NF-III (pmoles)
10 1 0.2 0.1 0.05 0.03 —
Protein-DNA complex
Free probe
1 2 3 4 5 6 7
B
100℃
34 17 8.5 4.25 2.12
NF-I= 3 pmoles
34 17 8.5 4.25 2.12 —DBP (pmoles)
d.s
s.s
1 2 3 4 5 6 7 8 9 10 11 12
NF-I (pmoles)
30 3 0.6 0.3 0.15 0.08 —
DNA-protein complex
Free probe
1 2 3 4 5 6 7

Chapter 3. Pyridoxal-5'-phosphate (PLP) inhibition of the Adenovirus type 5 DNA polymerase.

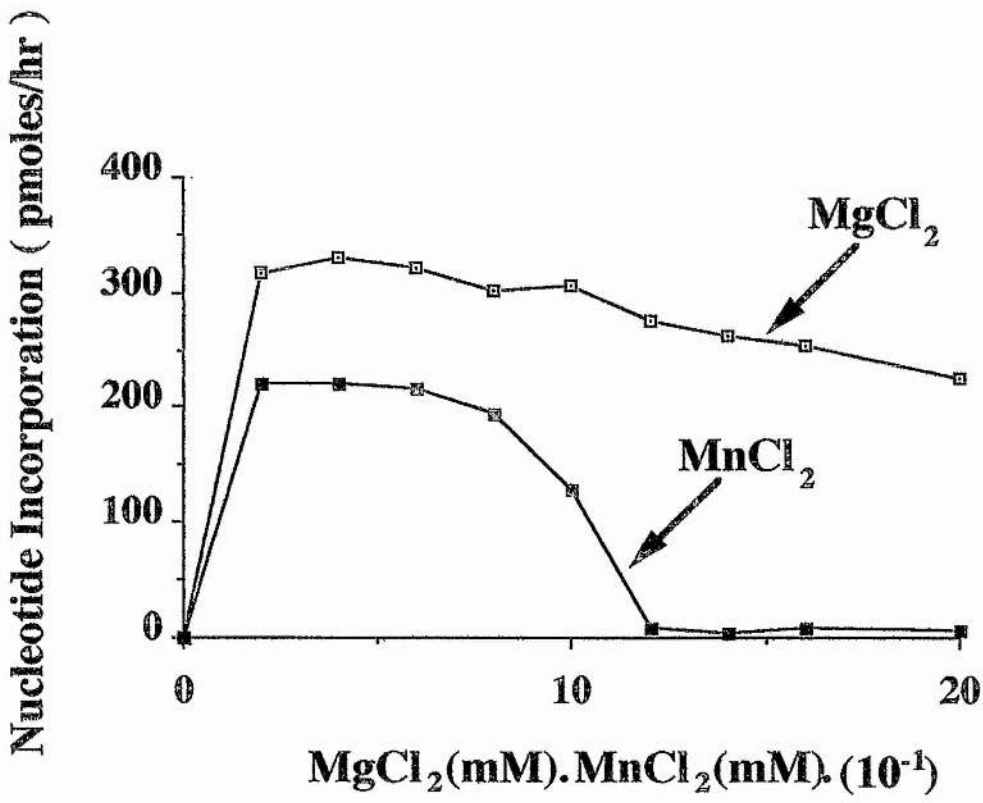
Previous studies on Ad.2 DNA polymerase from adenovirus infected Hela cells has provided the basis of most of the characterisation on the enzyme (Field et al., 1984). Extensive sequence homology studies between both eukaryotic and prokaryotic DNA polymerases (both viral and cellular) has indicated the presence of a putative "active site" region in the C-terminal region of Ad.2 DNA polymerase (Larder et al., 1987). The role of this region in catalysis of the related Ad.5 DNA polymerase was examined through modification of the active site and inhibition of catalysis by pyridoxal-5'-phosphate (PLP).

3.1 Characterisation of recombinant baculovirus expressed Ad.5 DNA polymerase.

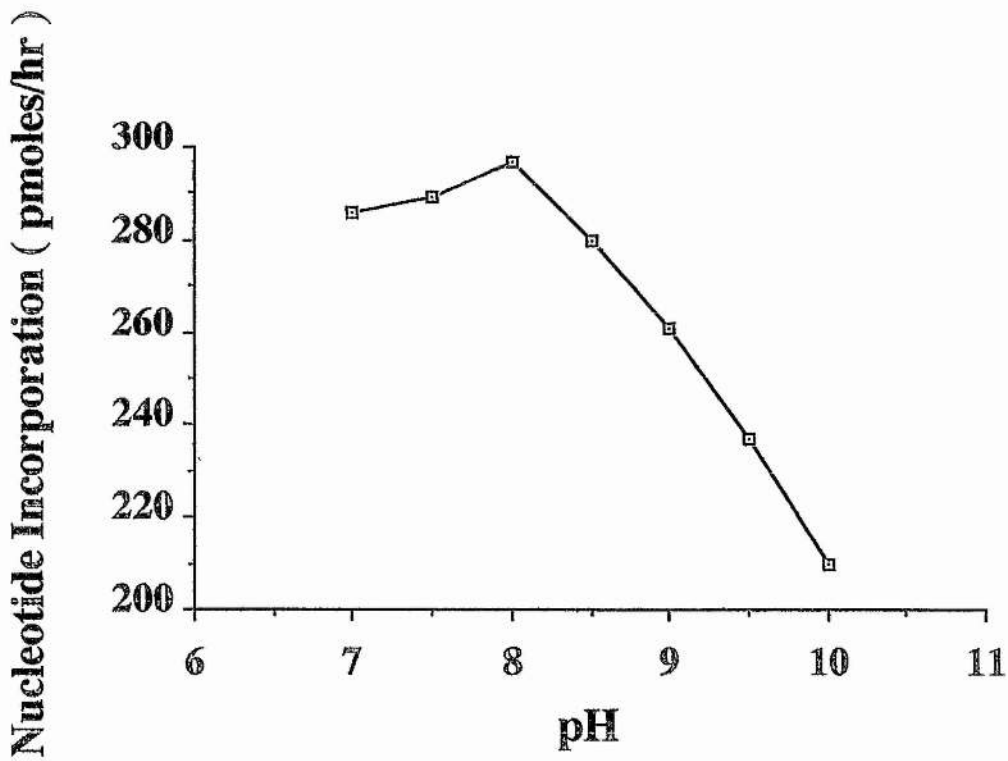
Previous studies on the Ad.2 DNA polymerase from Hela infected cells demonstrated that optimal DNA polymerase activity, using activated DNA as a substrate, required the presence of all four deoxyribonucleoside triphosphates, DNA and Mg^{2+} salt (Lichy et al., 1982 ; Field et al., 1984). Initial investigations on the baculovirus recombinant Ad.5 DNA polymerase therefore concentrated on establishing the conditions for optimal activity. A divalent metal cation (figure 3.1A) was absolutely required for activity; the optima for $MgCl_2$ and $MnCl_2$ being 5mM and 0.5mM, respectively. The optimum pH (figure 3.1B) in 50mM Tris-HCl buffers was 8.0, the reaction rates at pH 7 and 9 being 97 and 88%, respectively, of the rate at the optimum.

Figure 3.1 Dependence of Adenovirus DNA polymerase activity on divalent metal cation and pH. A. 1.5μg of Ad. 2 DNA polymerase incubated in the presence of the indicated concentrations of $MgCl_2$ and $MnCl_2$ as described in Materials and Methods, **B.** in the presence of Tris-HCl buffers varying from pH 7-10. All reactions were carried out in triplicate and a mean value obtained.

A.


Nucleotide Incorporation (pmoles/hr)
400
300
200
100
0
MgCl2
MnCl2
0
10
20
MgCl2(mM).MnCl2(mM).(10-1)

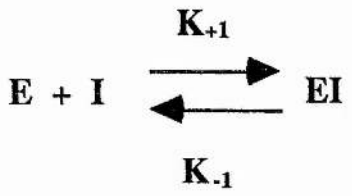
B.


Nucleotide Incorporation (pmoles/hr)
300
280
260
240
220
200
6
7
8
9
10
11
pH

3.2 Kinetics of Pyridoxal-5'-phosphate (PLP) inhibition of Ad.5 DNA polymerase.

Identification of amino acid side-chains involved at the catalytic centre of many enzymes has relied upon the use of chemical modifiers of amino acids, whose use is governed by two requirements: A. modification should be carried out under mild conditions; and B. only one type of amino acid group must be modified at a time (for review see, Price and Stevens, 1989). The catalytic site of Ad.5 DNA polymerase was investigated using the chemical modifier Pyridoxal-5'-phosphate (PLP), a naturally occurring cofactor which has been shown to be an effective inhibitor of many enzymes which have binding sites for nucleoside triphosphate e.g. Reverse Transcriptase (Basu et al, 1989), DNA polymerase α from Drosophilia melanogaster (Diffley, 1988) and E.coli DNA polymerase 1 (Basu and Modak, 1987). PLP inhibits through the chemical modification of lysine or ariginine amino acid side-chains. It contains a phosphate moiety important in the initial binding of the inhibitor at the phospho-ligand binding site, while the formyl moiety it contains at carbon position 4 is required for the formation of a covalent imine or Schiff base with a primary amino group, usually the ε-amino group of lysine at this binding site. The initial covalent imine bond it forms is readily hydrolyzable and reversible in aqueous solution and needs to be reduced to a stable, secondary amine by the addition of at least a ten-fold excess of sodium borohydride ($NaBH_4$). Inhibition of Ad.5 DNA polymerase with a molar excess of PLP should normally

follow pseudo-first order kinetics;



E=Enzyme
I=Inhibito r

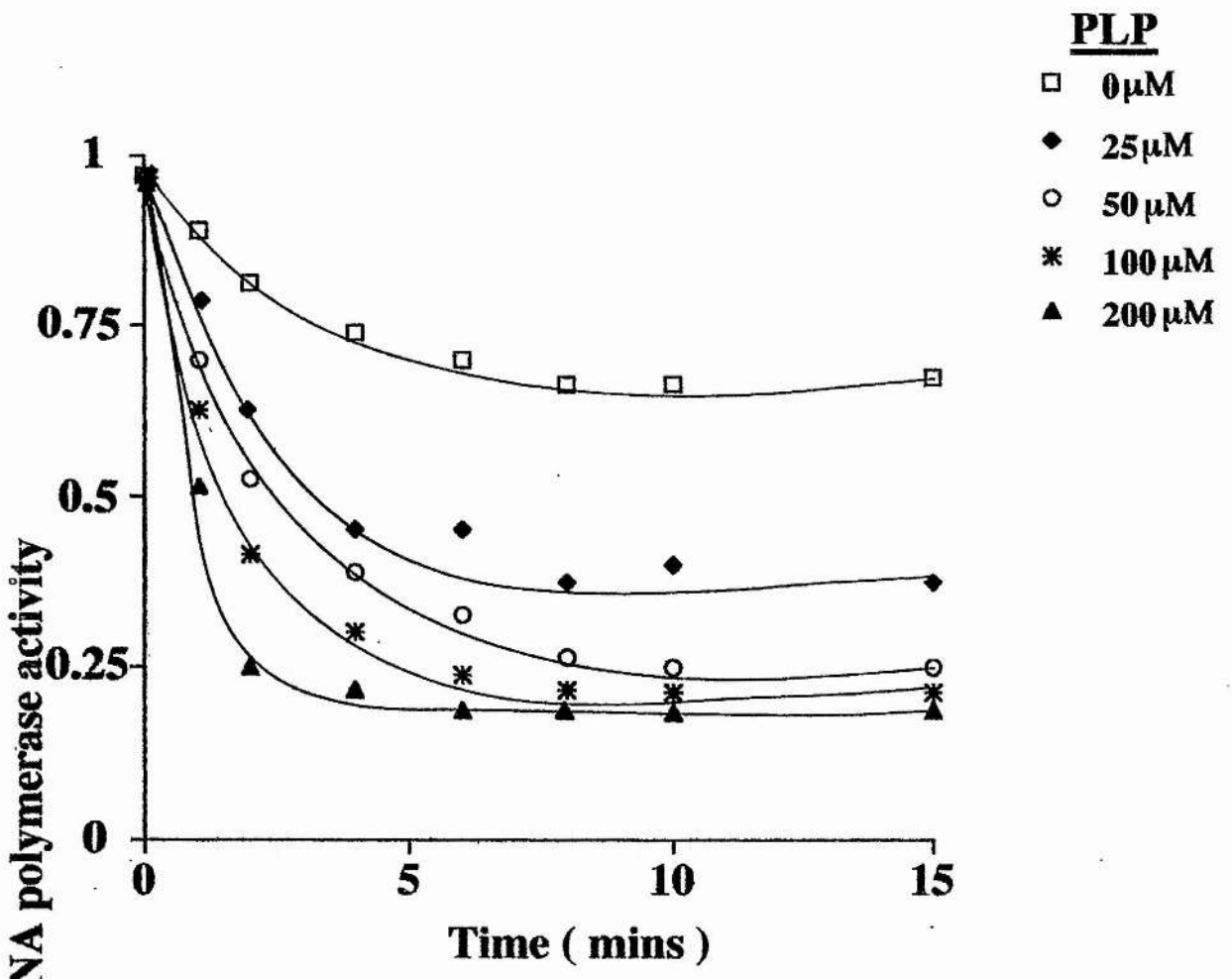
EI=Enzyme-Inhibitor Complex

with the apparent first order forward rate constant defined as K_{+1}; expected to increase linearly with increasing inhibitor concentration. However, when an inhibitor binds to a specific site on an enzyme, apparent first order rate constants plateau at higher inhibitor levels, exhibiting a saturation effect (Meloche et al, 1967). This is a characteristic property of an affinity label, indicating an initial, non-covalent interaction between the inhibitor and a specific site on an enzyme prior to the formation of a stable covalent bond. In the case of PLP inhibition, a reversible imine bond is formed prior to being reduced by sodium borohydride, forming a stable irreversible amine bond.

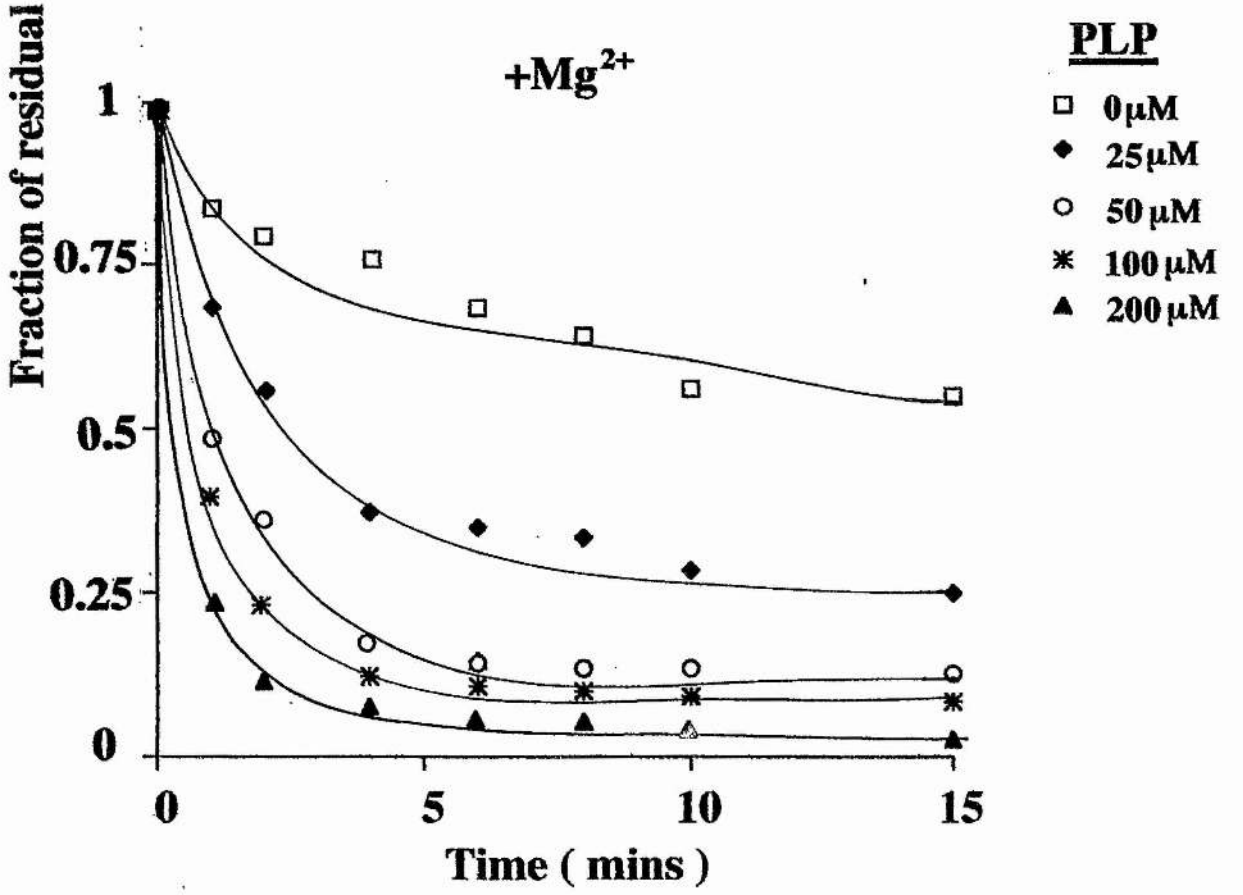
Ad.5 DNA polymerase was incubated in the absence of substrates with various concentrations of PLP in the presence (figure 3.2A) and absence (figure 3.2B) of $MgCl_2$. Data from figure 3.2A demonstrates that Ad.5 DNA polymerase is inhibited in a time-dependent manner, reaching a limit after several (8-12) mins. Apparent first order rate constants for inhibition at each PLP concentration, in both the presence and absence of Mg^{2+}, were calculated and are shown in figure 3.2C. The results in figure

Figure 3.2 Kinetics of PLP inhibition of Adenovirus type 5 DNA polymerase. A. 43µg (total) of purified Ad. 5 DNA polymerase incubated with PLP at final concentrations of 0, 25, 50, 100, 200µM in the presence of 20mM potassium bicine, pH 8, 10% glycerol and 1mM EDTA, pH 8 in a final volume of 400µl. At the times indicated, 40µl aliquots were removed and reduced with 10µl of 15mM $NaBH_4$. After incubation at 0°C for 10 minutes, aliquots were assayed for residual DNA polymerase activity with activated DNA as described under Materials and Methods and compared to reactions treated identically, except containing no PLP, **B.** reactions were as above except in the absence of $MgCl_2$, **C.** values of K (mins) for PLP inhibition were determined from data in A and B. Rate values were calculated by measuring the gradient of the initial rate of the reaction slopes. All reactions were carried out in triplicate and a mean value obtained.

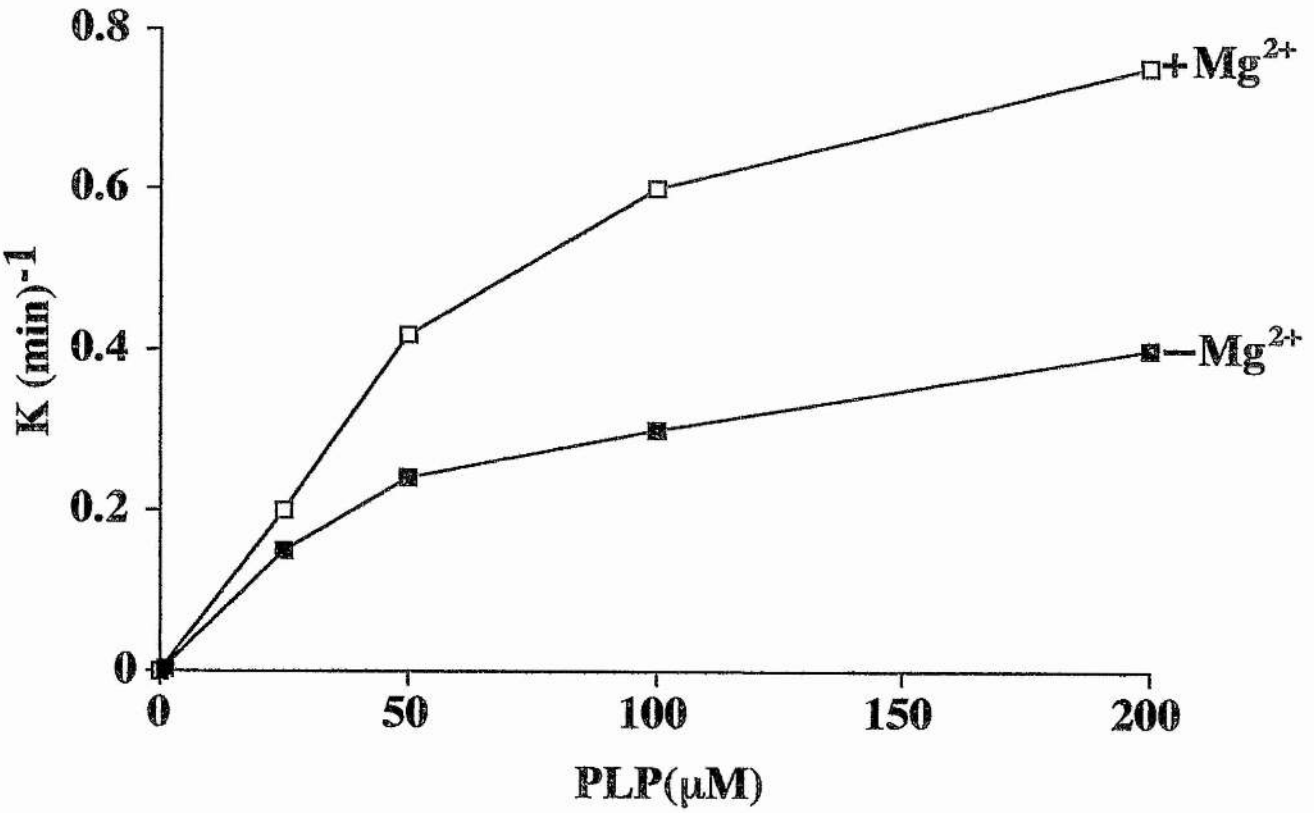
A.


PLP
0μM
25μM
50μM
100μM
200μM
1
0.75
0.5
0.25
0
0
5
10
15
Time (mins)
Fraction of residual DNA polymerase activity

B.


+Mg2+
PLP
0μM
25μM
50μM
100μM
200μM
1
0.75
0.5
0.25
0
0
5
10
15
Time (mins)
Fraction of residual DNA polymerase activity

C.



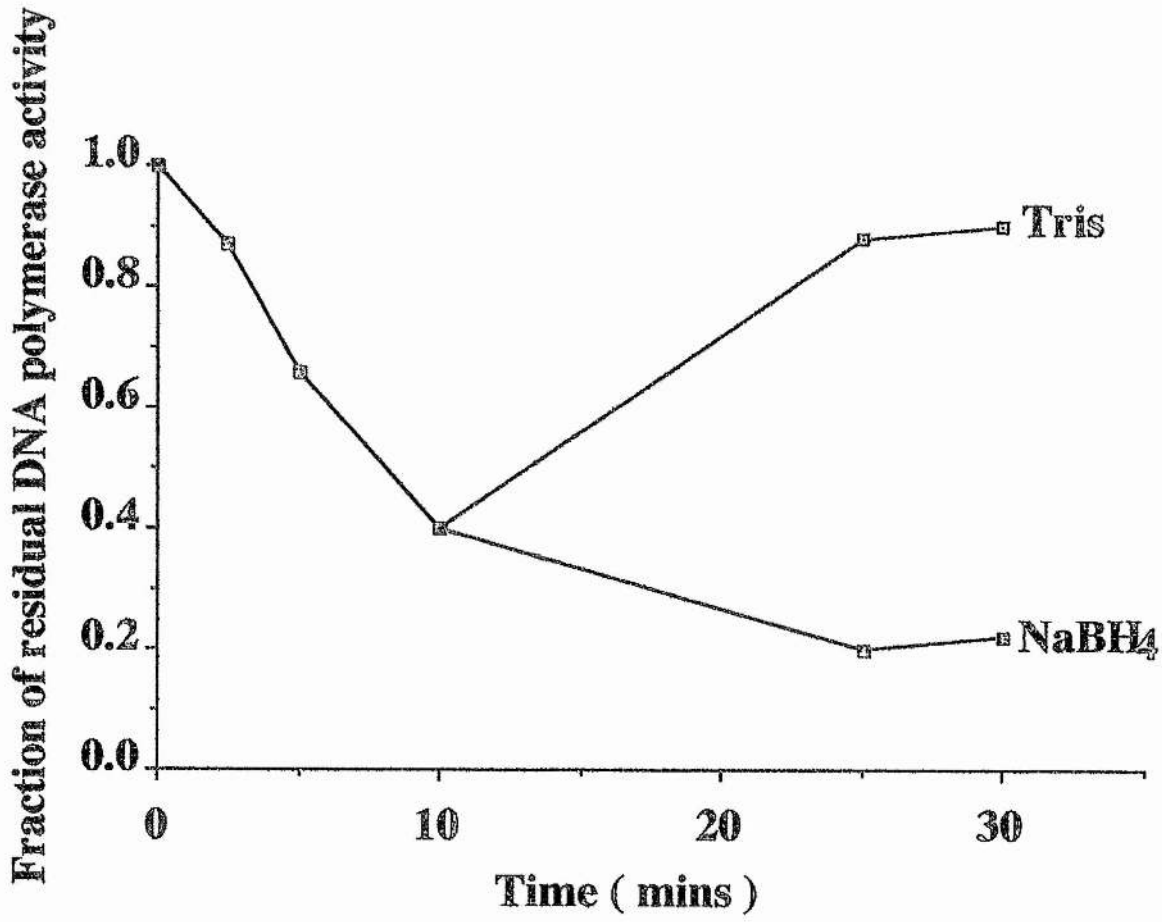
3.2C demonstrate that PLP inhibition of the polymerase probably occurs through binding to a specific site(s) on the enzyme in both the presence and absence of Mg^{2+}. The presence of Mg^{2+} also increases the ability of PLP to inhibit Ad.5 DNA polymerase activity.In fact, Mg^{2+} has been shown to have multiple roles in DNA polymerase reactions, which include the binding of both primer (Fisher and Korn, 1981) and dNTP's (Mildvan and Loeb, 1979) to the active site, therefore its not surprising that Mg^{2+} stimulates the binding of PLP to the enzyme. This Mg^{2+}- PLP chelate has been shown to be required for effective inhibition of other DNA polymerases (Basu and Modak, 1987 ; Basu et al., 1988).

3.3 Reversibility and pH dependence of PLP inhibition.

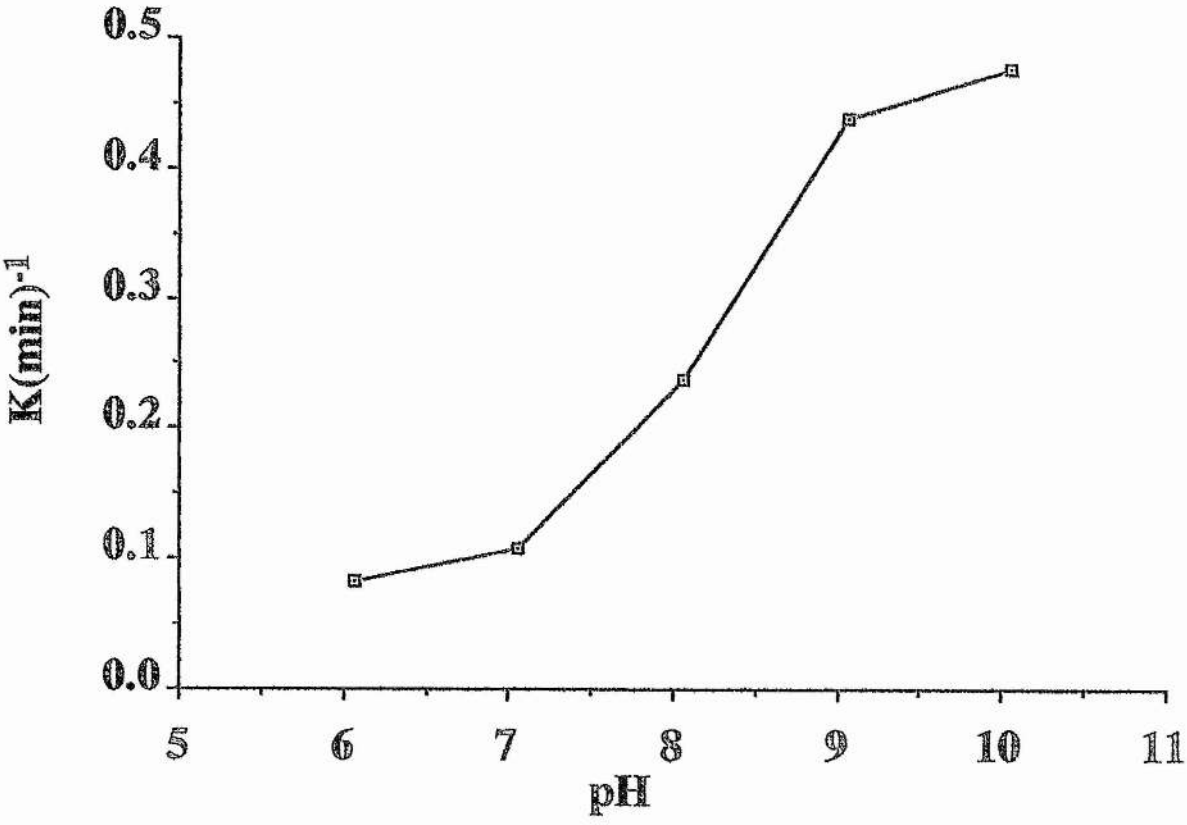
To demonstrate that PLP inhibition of Ad.5 DNA polymerase occurs via reversible imine bond formation, which can be rendered irreversible by reduction with $NaBH_4$, use was made of the ability of primary amines such as Tris to inactivate free PLP via imine bond formation . After PLP inhibition of Ad.5 DNA polymerase was allowed to approach equilibrium (10 mins), the reaction mixture was split in half. One half was treated with an excess of Tris and then reduced with $NaBH_4$. The other half was reduced by $NaBH_4$ addition and then treated with excessTris and assayed for residual DNA polymerase activity. These were compared to reactions containing no PLP. Figure 3.3A demonstrates that the addition of Tris before reduction can reverse inhibition, while addition of Tris after reduction cannot reverse inhibition. Therefore it would seem that PLP inhibition of Ad.5 DNA polymerase activity occurs by reversible imine bond formation. During PLP inhibition this would involve nucleophilic attack by a nonprotonated amino group on the carbonyl group of PLP. This type of attack should cause a sharp rise in the rate of inhibition at some pH value, which can then be used to infer the pKa of the amino

Figure 3.3 Reversibility and pH dependence of PLP inhibition.
A. 100μg (total) of Ad.5 DNA polymerase incubated in 200μl with 50μM PLP, 20mM potassium bicine, pH 8, 10% glycerol and 1mg/ml BSA. At the times indicated aliquots (20μl) were withdrawn, reduced with 10μl of 15mM $NaBH_4$ and assayed for residual DNA polymerase activity using activated DNA. These were compared to control reactions treated identically except without PLP. After 12min, the reaction was split in half. To half Tris-HCl, pH 8 was added to 100mM, to the other, $NaBH_4$ was added to 1mM. After 20 mins, 1mM $NaBH_4$ was added to the first half and 100mM Tris-HCl, pH 8 was added to the second half; **B.** 60μg (total) of purified Ad.5 DNA polymerase incubated with 100μM PLP in 25mM potassium bicine at the indicated pH values in 280μl. At 0, 1, 2, 4, 8 and 10 mins, 40μl aliquots were withdrawn, reduced with 2mM $NaBH_4$ and assayed on activated DNA for residual DNA polymerase activity. Reactions identical to the above, except without PLP, were used as controls. Rate values were calculated by measuring the gradient of the initial rate of the reaction slopes. $K(mins)^{-1}$ represent the initial rates of inhibition with PLP. All reactions were carried out in triplicate and a mean value obtained.

A.


Fraction of residual DNA polymerase activity
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (mins)
Tris
NaBH4

B.


K(min)-1
0.5
0.4
0.3
0.2
0.1
0.0
5
6
7
8
9
10
11
pH

group on the polymerase which forms the imine bond with PLP (Bull et al, 1975). Rate constants (K) for PLP inhibition of Ad.5 DNA polymerase were determined over a range of pH values. There is a sharp increase in the rate (K) between pH 7-9. An increase in the rate of PLP inhibition was seen for both E.coli RNA polymerase and DNA polymerase α between pH 7.8 and 8.2 and was interpreted to mean that the critical amino group exhibited a pKa between 7.8 and 8.2 (Bull et al., 1975 ; Diffley, 1988). In both cases the amino group was shown to be the ε-amino group of a lysyl residue. Since the ε-amino group of free lysine has a much higher pKa (pKa=10.79), it was suggested that this lysyl residue was unusually reactive and, therefore, probably in the active site of the enzyme. This could be the case with Ad.5 DNA polymerase as its rate of inhibition is highest between the to pH values above. Although increases in rate constants have been used to pinpoint crucial residues, it must be stressed that possible conformational changes in this pH range could artificially increase the rate of noncovalently binding of PLP to the polymerase and therefore an increase in covalent bond formation. However, the optimum pH for Ad.5 DNA polymerase, which is pH 8.0, is within the pH range were the rate of reaction increases.

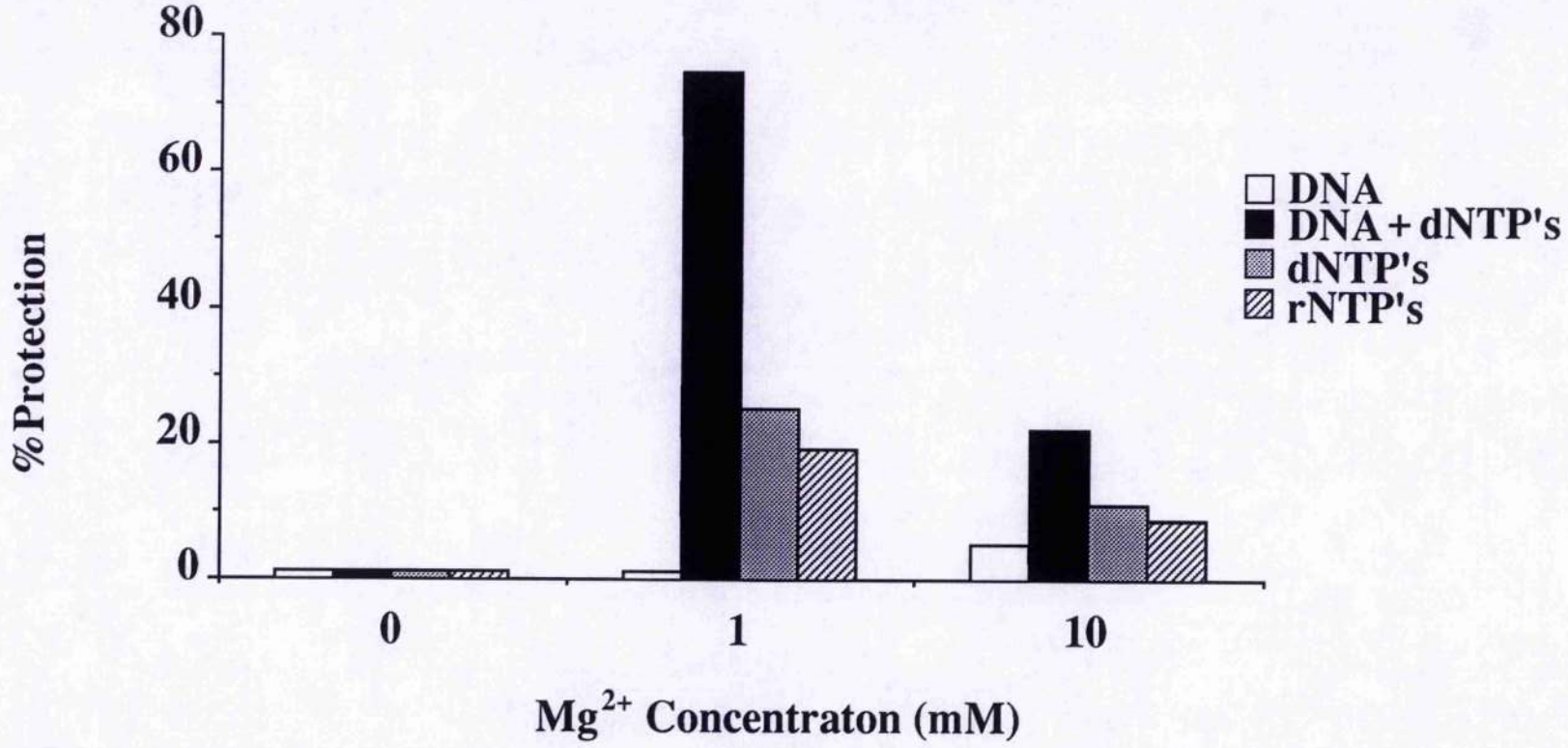
3.4 Effect of substrates on PLP inhibition.

If PLP inhibition of Ad.5 DNA polymerase is occurring via covalent bond formation at a substrate binding pocket, then occupation of this site by appropriate substrates should block PLP inhibition. This approach has been used to investigate, in detail, the Human immunodeficiency virus reverse transcriptase (Basu et al., 1988), Murine Leukemia virus reverse transcriptase (Basu et al., 1989) and Drosophilia melanogaster DNA polymerase α (Diffley et al., 1988) activities. All of these enzymes seem to obey a rigidly ordered substrate binding mechanism, with a single stranded DNA template binding first, followed by the binding of the correct deoxyribonucleoside triphosphate. Two template-primer / dNTP binding systems were chosen to look at the substrate binding mechanism of Ad.5 DNA polymerase. In the first system, Ad.5 DNA polymerase was incubated in the presence and absence of activated DNA and all four dNTP's (as template-primer and nucleotide source, respectively) at three different Mg^{2+} concentrations, in the presence of 50μM PLP. After incubation at 37°C for 15 mins the reaction mixtures were treated with an excess of $NaBH_4$, left to cool on ice, then assayed for residual DNA polymerase activity (see Materials and Methods) and compared with reactions which had no PLP added (figure 3.4). Percentage protection is defined as the percent of Ad.5 DNA polymerase activity protected by the indicated substrates (Material and Methods). The results from figure 3.4 demonstrate that activated DNA alone affords little or no protection , whereas dNTP's alone affords protection only at 1mM $MgCl_2$ (25%) and 10mM $MgCl_2$ (11%), respectively. Ribonucleoside triphosphates (rNTP's) were found to have the same basic pattern of protection as for dNTP's, the highest amount of protection occurred at 1mM $MgCl_2$ (19%), with reduced protection occurring at 10mM (9%) and no protection in the absence of $MgCl_2$. Protection by dNTP and rNTP is optimal at a low

Figure 3.4 Protection from PLP inhibition by various substrates. 10μg of purified Ad.5 DNA polymerase was incubated with 50μM PLP and the indicated concentrations of $MgCl_2$ in the presence or absence of various nucleoside triphosphates and activated DNA in 25mM potassium bicine, pH 8, then reduced with an excess of $NaBH_4$. Activated DNA and dNTP's were added to a final concentration of 1mg/ml and 1mM, respectively. After 15 mins at 37°C, 10μl aliquots from each reaction were assayed for residual DNA polymerase activity as described under Materials and Methods and compared to identical reactions containing no PLP.All reactions were carried out in triplicate and a mean value obtained. Percent protection is defined as:

(Fraction of residual activity with substrates-fraction of residual activity without substrates) x 100.

(1-Fraction of residual activity without substrates)


80
60
40
20
0
% Protection
0
1
10
Mg2+ Concentraton (mM)
DNA
DNA + dNTP's
dNTP's
rNTP's

Mg^{2+} concentration and is probably due to PLP being less inhibitory as a result of the substrates competing for the Mg^{2+} ions in the reaction. Protection was highest when both DNA and dNTP's were present at 1mM $MgCl_2$ (78%),with this figure dropping to 20% and 0% at 10mM and 0mM $MgCl_2$, respectively. Therefore the ability of template-primer and dNTP to protect Ad.5 DNA polymerase from PLP seems to be dependent upon the Mg^{2+} concentration. This can be demonstrated in a defined system using poly (dA).oligo $(dT)_{12-18}$ as the template-primer, (table 3.1). Reactions were carried out at a Mg^{2+} concentration of 0.1mM (10% of the optimal). Under these conditions, dTTP in the presence of poly (dA).oligo $(dT)_{12-18}$ is now seen to offer a significant amount of protection from PLP inhibition, (85%) while unmatched nucleotides, (30%, 24%, 26%, respectively for dGTP, dCTP, dATP) offer no more protection than primer-template alone (24%). Nucleotide alone (dTTP) showed little protection (12%). The values for percent protection (at these low Mg^{2+} concentrations) probably represent a slight underestimate of the actual protection since they were compared to control reactions without Mg^{2+}. This was done to ensure that protection is not a result of the chelation of free Mg^{2+} by the substrates which would render PLP less inhibitory. These results suggest that (at suboptimal Mg^{2+} concentrations) template-primer and dNTP can bind in a potentially productive manner, but some aspect of catalysis is slowed, leaving the substrates in an abortive ternary complex which can protect the polymerase from PLP inhibition. To test this theory and to eliminate the possibility that PLP inhibited in a qualitatively different manner at low and high Mg^{2+} concentrations, the ability of a true dead-end ternary complex to protect Ad.5 DNA polymerase at two $Mg^{2+}$was tested. Early characterisation studies on Ad.2 DNA polymerase (Lichy et al., 1982) had shown that the addition of a ddNTP inhibited almost 100% of the

Table 3.1. Protection from PLP inhibition by substrates in a defined system. 10μg of Ad.5 DNA polymerase incubated with 50μM PLP, 25mM potassium bicine, pH 8, 0.1mM $MgCl_2$ in the presence of the indicated substrates. The final concentrations of each individual dNTP and poly (dA). oligo $(dT)_{12-18}$ were 1mM and 0.1mM , respectively. Residual polymerase activity was assayed as above. All reactions were carried out in triplicate and a mean value obtained.

TABLE 3.1

TEMPLATE-PRIMER	NUCLEOSIDE TRIPHOSPHATE	%PROTECTION
Poly(dA).oligo(dT)$_{12\text{-}18}$	NONE	24
NONE	dTTP	12
Poly(dA).oligo(dT)$_{12\text{-}18}$	dTTP	85
Poly(dA).oligo(dT)$_{12\text{-}18}$	dGTP	30
Poly(dA).oligo(dT)$_{12\text{-}18}$	dCTP	24
Poly(dA).oligo(dT)$_{12\text{-}18}$	dATP	26

Table 3.2. Protection from PLP inhibition by a ddNTP containing ternary complex. PLP inhibition and residual DNA polymerase activity assays were performed as in figure 3.4, except that only two $MgCl_2$ concentrations (1mM and10mM) were used. The concentration of nucleoside triphosphates and dideoxynucleoside triphosphates was 1mM. All reactions were carried out in triplicate and a mean value obtained.

TABLE 3.2

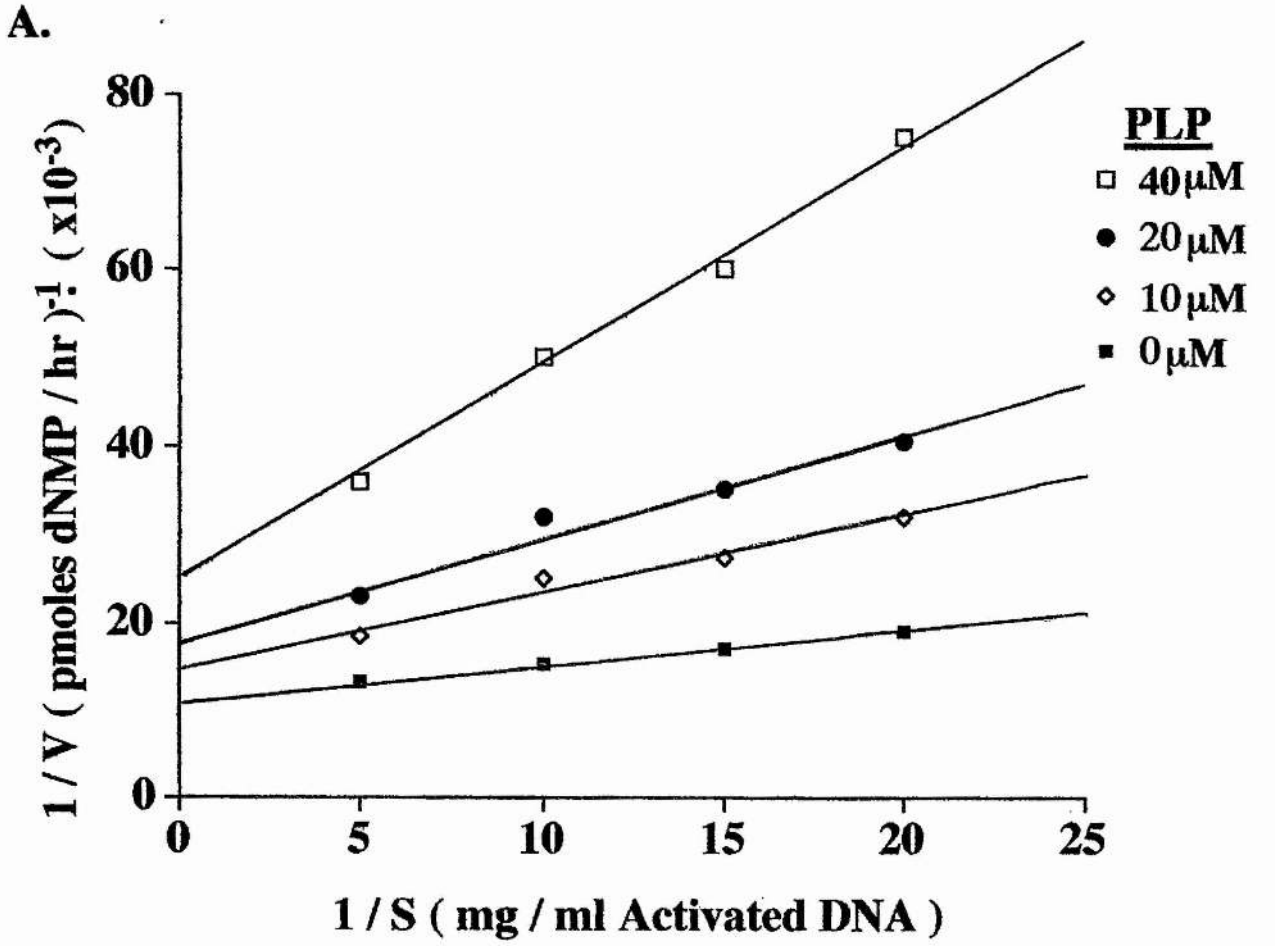
TEMPLATE PRIMER	NUCLEOSIDE TRIPHOSPHATE	PLP(μM)	$MgCl_2$(mM)	%PROTECTION
ACTIVATED DNA	dCTP dGTP dATP ddTTP	50	1 and 10	98
ACTIVATED DNA	dCTP dGTP dTTP ddATP	50	1 and 10	98
ACTIVATED DNA	dGTP dATP dTTP ddCTP	50	1 and 10	99
ACTIVATED DNA	dCTP dATP dTTP ddGTP	50	1 and 10	98

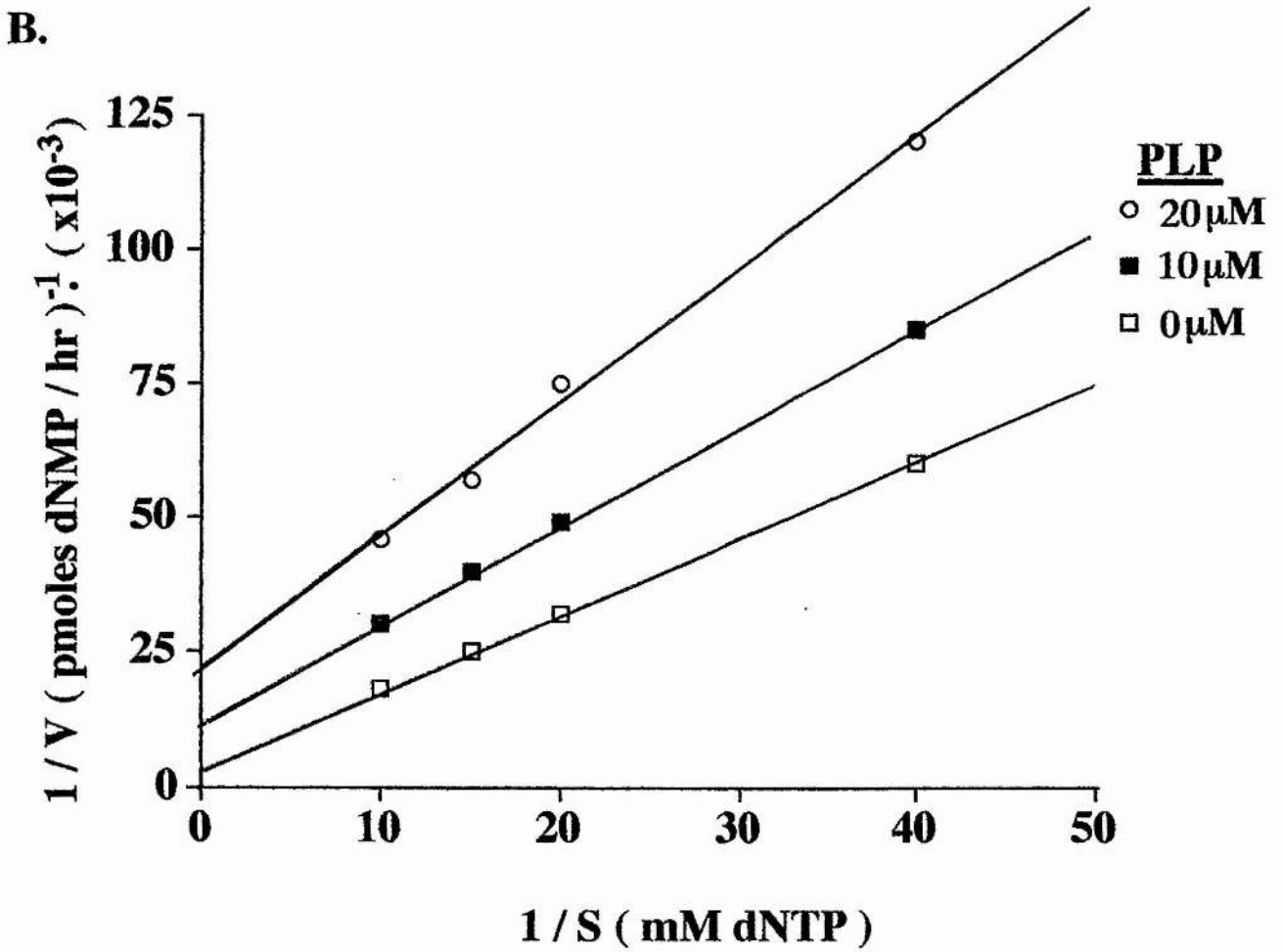
polymerase activity. The ability of ddNTP's to protect polymerase activity through competitive inhibition with PLP was studied more closely. To investigate the possibility that the site of ddNTP inhibition coincided with that for PLP inhibition, PLP was incubated in the presence and absence of activated DNA, 3xdNTP's and one ddNTP at both 1mM and10mM $MgCl_2$ (table 3.2). Addition of a ddNTP into the reaction at both Mg^{2+} concentrations resulted in nearly 100% protection . At the same Mg^{2+} concentrations, in the presence of dNTP's, protection was only 25% (10mM) and 75% (1mM) respectively (see figure 3.4). Thus, the presence of activated DNA plus three dNTP's, dATP, dCTP, dGTP and any ddNTP seems to block PLP inhibition by binding to the same site on Ad.5 DNA polymerase as the ternary complex formed with the normal template-primer and dNTP. Presumably the ddNTP is immediately incorporated into the growing DNA chain, resulting in the formation of a dead-end ternary complex.

3.5 Effect of PLP on initial reaction velocities.

Ad.5 DNA polymerase substrates at optimal Mg^{2+} concentrations are ineffective in blocking PLP inhibition. Two possibilities exist to explain this problem: firstly, Ad.5 DNA polymerase can only leave a ternary complex by two pathways; **A**, either backward by ordered release of substrates; **B**, forward by catalysis and translocation of the newly formed 3' end to the primer binding site and/or subsequent release of products. Secondly, since a dead-end ternary complex cannot follow this second pathway, a higher concentration of substrates may be required to occupy most of the polymerase as a ternary complex (therefore at infinite concentrations of one or all of the substrates, the normal ternary complex should protect from PLP inhibition); alternatively, a rate limiting step may exist in the polymerisation cycle between ternary complex formation

Figure 3.5 . Initial velocity analysis of PLP inhibition with respect to template-primer and dNTP concentration. A. Initial reaction velocities using the indicated concentrations of activated DNA as template-primer. 5μg of Ad.5 DNA polymerase, 10mM $MgCl_2$, 25mM potassium bicine, pH 8, in the presence of 0, 10, 20 and 40μM PLP, incubated at 37°C for 1, 2, 4, 6, 8 and 10 mins and stopped by the addition of cold TCA as described under Materials and Methods. **B.** initial reaction velocities were determined as above, except the dNTP concentration was varied (ratio of four dNTP's was maintained at 1:1:1:1) as indicated.The concentrations of PLP were the same as in figure 3.6 A, with the concentration of activated DNA constant at 10μg (total). All reactions were carried out in triplicate and a mean value obtained.


A.
80
60
40
20
0
1 / V (pmoles dNMP / hr)-1 (x10-3)
0
5
10
15
20
25
1 / S (mg / ml Activated DNA)
PLP
40μM
20μM
10μM
0μM


B.
125
100
75
50
25
0
1 / V (pmoles dNMP / hr)-1 (x10-3)
0
10
20
30
40
50
1 / S (mM dNTP)
PLP
20μM
10μM
0μM

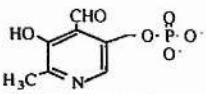
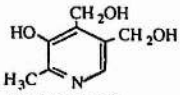
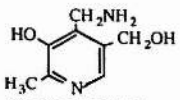
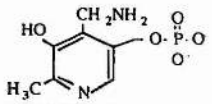
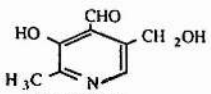
and the nucleotide binding step.Therefore, if PLP binds to Ad.5 DNA polymerase in an inhibitory fashion during this time, then even infinite concentrations of substrate will not protect from PLP inhibition. The two alternatives can be distinguished by looking at the effect PLP has on the initial velocities of Ad.5 DNA polymerase reactions. If the first of the possibilities is correct, then inhibition by PLP should be competitive with one or more of the substrates, while with the second possibility, inhibition by PLP, should not be competitive with any substrate. Initial reaction velocity studies using PLP have been carried out with a number of enzymes (Modak, 1976 ; Diffley, 1988). In figure 3.5 reciprocal plots show that PLP inhibition is not competitive with either: **A**, activated DNA or **B**, total dNTP (using activated DNA as a template-primer). This is consistent with the results presented in figure 3.4, in which both template-primer and dNTP's alone were ineffective in protecting the polymerase from PLP inhibition.The kinetic data above suggests that the second possibility most accurately describes the PLP inhibition of Ad.5 DNA polymerase. That is, only the template-primer and dNTP ternary complex can protect the polymerase from PLP inhibition, with PLP only capable of inhibiting the polymerase at a rate limiting step in the polymerisation reaction occurring after the formation of the ternary complex and before the binding of the next nucleotide.

3.6. Specificity of PLP inhibition.

In order to determine the specificity of inhibition by pyridoxal-5'-phosphate, four other chemically related compounds were tested for inhibitory effects on Ad.5 DNA polymerase (table 3.3). The results indicate that the pyridoxine derivatives, pyridoxine, pyridoxal, pyridoxamine and pyridoxamine-5'-phosphate (at a concentration of100μM) have no effect on the DNA synthesis of Ad.5 DNA

Table 3.3. Effects of various pyridoxine derivatives on the activity of Ad. 5 DNA polymerase. 3μg of purified Ad.5 DNA polymerase incubated with the indicated concentrations of pyridoxine derivatives. After incubation at 37°C for 1 hour, reactions were stopped by the addition of cold TCA and DNA polymerase activity measured as described in Materials and Methods. All reactions were carried out in triplicate and a mean value obtained.

Table 3.3

PYRIDOXINE DERIVATIVE(100uM)	NUCLEOTIDE INCORPORATION (pmol/hr)	%INHIBITION
NONE	77	0
 **PYRIDOXAL-5-PHOSPHATE**	26	67
 **PYRIDOXINE**	77	0
 **PYRIDOXAMINE**	75	2
 **PYRIDOXAMINE-5-PHOSPHATE**	75	2
 **PYRIDOXAL**	73	5

polymerase. Pyridoxal-5'-phosphate at the same concentration inhibited inhibition by up to 67%. PLP inhibition of Ad.5 DNA polymerase therefore seems to be highly specific.

3.7. Ad.5 DNA polymerase associated nuclease activity.

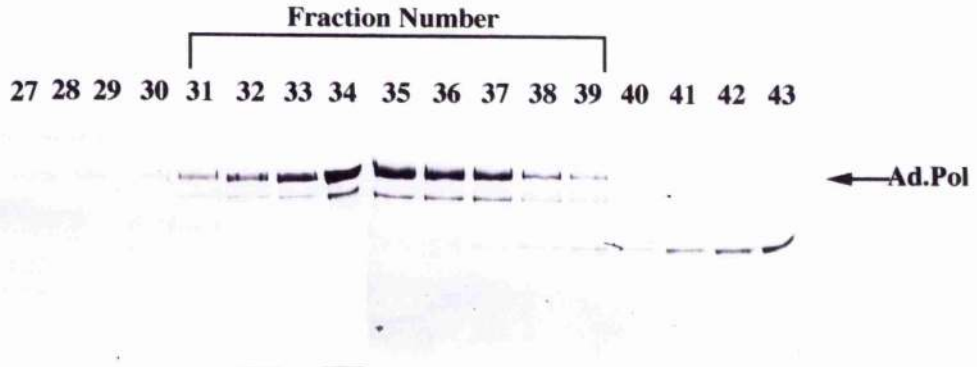
Previous studies had shown that a 3'-5' exonuclease activity is associated with the Ad.2 DNA polymerase (Field et al, 1984). To test whether recombinant baculovirus Ad.5 DNA polymerase contained this same activity, a preparation of Ad.5 DNA polymerase was fractionated by glycerol gradient centrifugation. Fractions were collected, analysed by SDS PAGE (figure 3.6A) and DNA polymerase and exonuclease activities determined (figure 3.6B). A contaminating band present just below the 140KDa DNA polymerase in the glycerol gradient fractions which corresponds to proteolysed polymerase was also found to be present. Alignment of the fractions from the glycerol column with optimal polymerase and exonuclease activities indicated that fractions 31-40 (F31-40) contained both activities. The results indicate that baculovirus derived Ad.5 DNA polymerase contains an extrinsic exonuclease activity which co-sedimentates with the DNA polymerase activity.

3.8. Effect of PLP on Ad.5 DNA polymerase and its associated 3'-5' exonuclease activities.

Sequence homology studies have indicated that the DNA polymerase and exonuclease activities of α-like DNA polymerases (of which Ad.5 DNA polymerase is a member) are located on different domains of the protein. To test whether the two functions shared the same catalytic site, purified Ad.5 DNA polymerase was incubated with 0, 25, 50, 100, 150, 200μM PLP (figure 3.7) The results show a progressive decrease in the

Figure 3.6 Co-sedimentation of an 3'-5' exonuclease activity with the 140KDa Adenovirus 2 DNA polymerase. 300μg of purified Ad.5 DNA polymerase loaded onto a native 5%-30% glycerol gradient and centrifuged at 49,000rpm on an SW40i rotor for 4 hours. Fractions were collected and analysed by SDS PAGE (A) and assayed for exonuclease and polymerase activities (B) as described in Materials and Methods. All reactions were carried out in triplicate and a mean value obtained.

A.


Fraction Number
27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43
Ad.Pol

B.

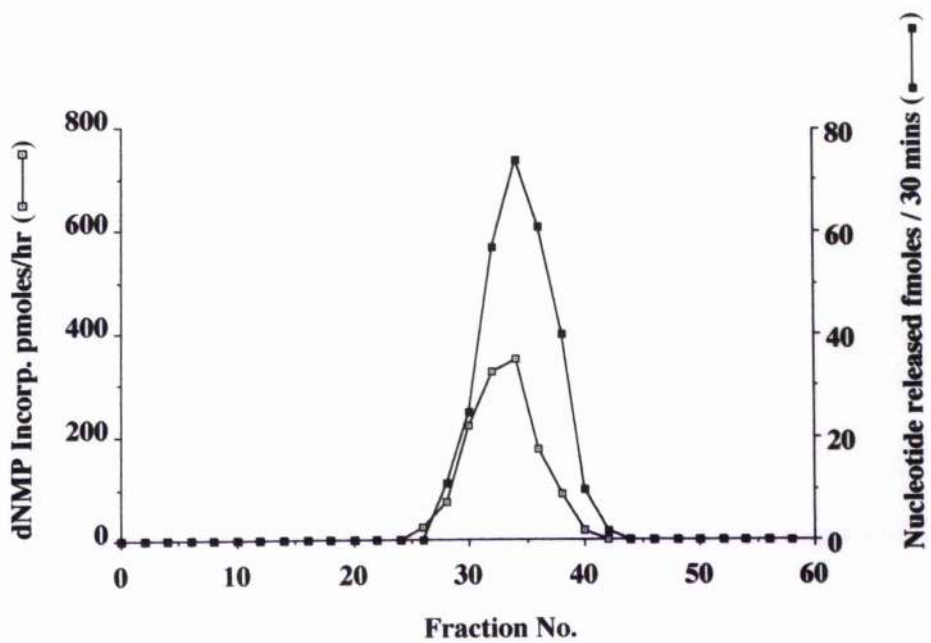
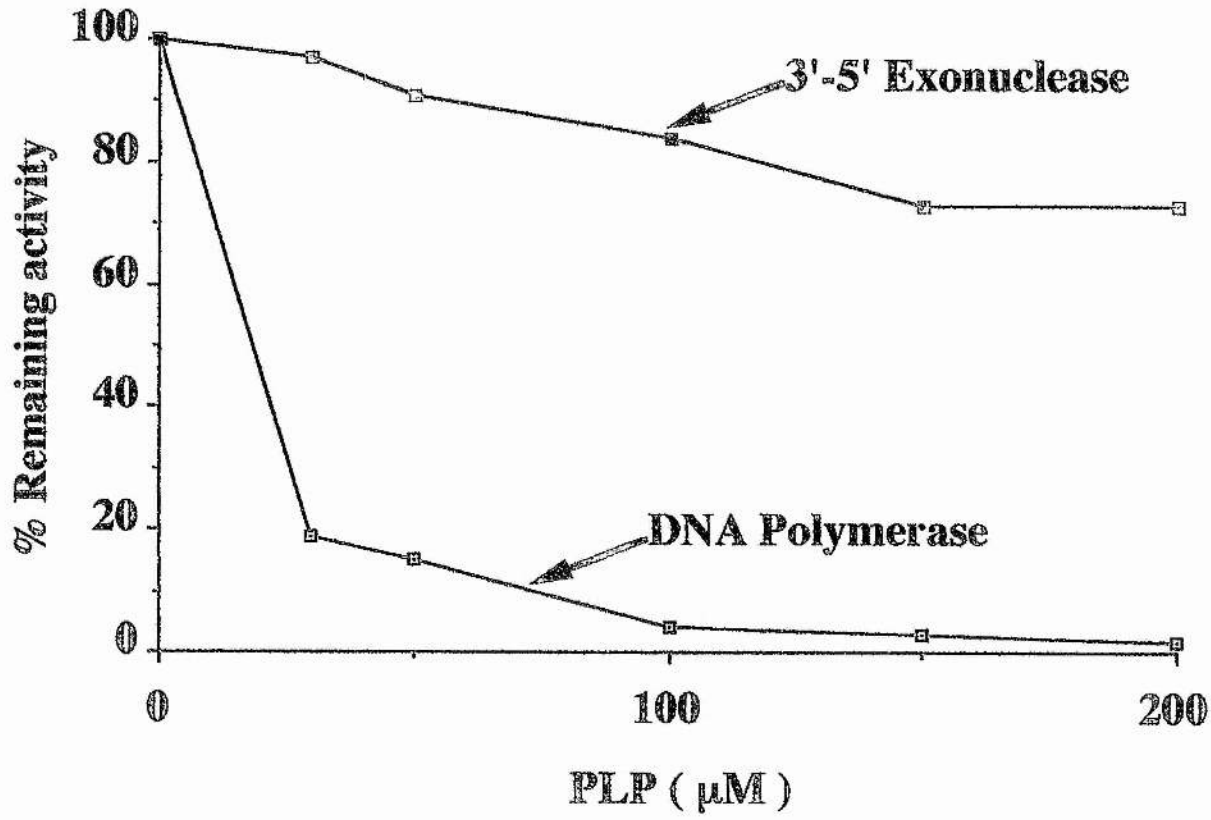

dNMP Incorp. pmoles/hr (□—□)
Nucleotide released fmoles / 30 mins (■—■)
800
600
400
200
0
80
60
40
20
0
0
10
20
30
40
50
60
Fraction No.

Figure 3.7 Effect of pyridoxal-5-phosphate (PLP) on the DNA polymerase / exonuclease activities of Ad.5 DNA polymerase. 17μg Ad.5 DNA polymerase was incubated with the indicated concentrations of PLP in 50mM potassium bicine, pH 8, 10mM $MgCl_2$ at 37°C for 15 mins. The reaction mixtures were slowly cooled to O°C and a chilled solution of $NaBH_4$ added to give a final concentration of 2mM. After 15 mins on ice a small aliquot (20μl) was assayed for polymerase and exonuclease activities. Percentage inactivation for the Ad.5 DNA polymerase and exonuclease activities are shown. All reactions were carried out in triplicate and a mean value obtained.


100
80
60
40
20
0
% Remaining activity
3'-5' Exonuclease
DNA Polymerase
0
100
200
PLP (μM)

polymerase activity with increasing PLP concentrations, while exonuclease activity was not significantly affected. These results indicate that the DNA polymerase and exonuclease domains of Ad.5 DNA polymerase are likely to be on different parts of the protein.

3.9. PLP inhibition of the transfer of dCMP to pTP.

Previous studies had pointed to a direct transfer mechanism being involved in the transfer of dCMP onto pTP, the first residue in the nascent DNA chain, (Lichy et al, 1982). The transfer is thought to directly involve the adenovirus DNA polymerase whilst in the pTP-Pol heterodimer complex. To demonstrate whether the dCMP transfer and catalytic sites were one and the same, PLP inhibition of both Ad.5 DNA pol and pTP was carried out before introduction into the standard initiation assay. Figure 3.8 (lanes 1-5) demonstrates that PLP (present at 25, 50,100μM) inhibition of the Ad.5 DNA polymerase before addition to the in vitro initiation assay results in a noticeable decrease in the level of the 80KDa pTP-dCMP protein complex. Inhibition of pTP, however, shows no affect on the level of the 80KDa pTP-dCMP complex, (lanes 6-9). Therefore, PLP seems to inhibit pTP-dCMP complex formation only when the polymerase component of the heterodimer has been previously inhibited. This result, when taken in conjunction with the kinetic results above, suggests that PLP is inhibiting at a site common to both the catalytic and dCMP transfer activities of the Ad.5 DNA polymerase.

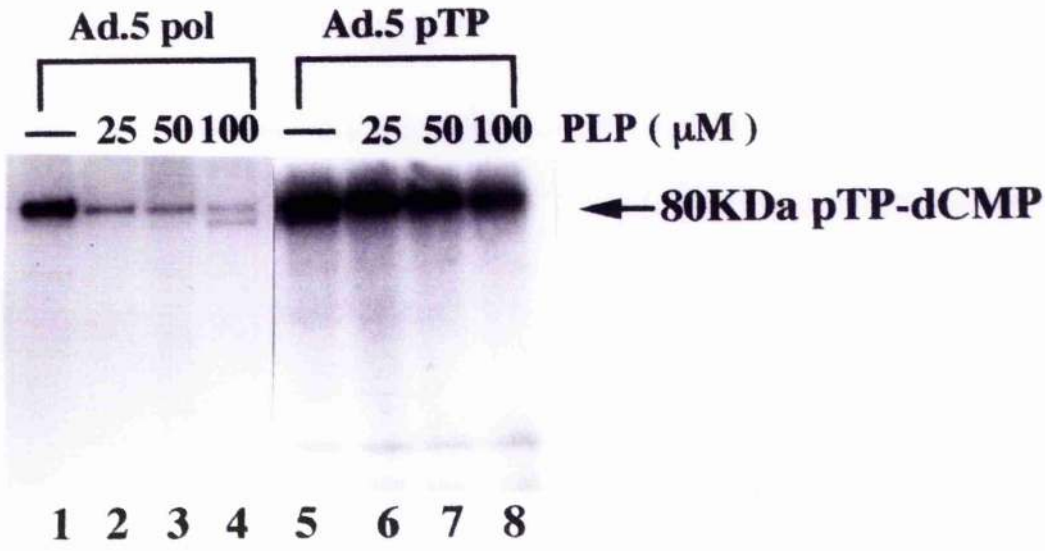


Figure 3.8. Inhibition of dCMP transfer by PLP. Purified Ad.5 DNA polymerase and preterminal protein were incubated with 0, 50, 100 and 200μM PLP (as indicated) at 37°C for 15 mins. PLP was reduced by the addition of cold $NaBH_4$ (final concentration of 2mM). The reaction mixture was left at O°C for an additional 15 mins before addition into the standard in vitro initiation assay (Material and Methods). The formation of the pTP-dCMP 80KDa complex, indicative of initiation was visualised as described under Materials and Methods.

Chapter 4. Attempted demonstration of the presence of reactive lysine(s) in the putative Ad.5 DNA polymerase active site.

The results presented in chapter 3 demonstrated that pyridoxal-5'-phosphate (PLP) inhibited the catalytic (polymerisation) function of the DNA polymerase at its active site. To identify the targets of pyridoxylation and to map the regions containing the putative lysine residue(s) involved in dNTP binding to the active site, differential labelling with tritiated sodium borohydride followed by comparative tryptic mapping using reverse-phase HPLC was performed.This approach had been used successfully to investigate important lysine residues involved in catalysis of various DNA polymerases (Modak et al, 1976; Basu and Modak, 1987).

4.1 ^{3}H Sodium borohydride affinity labelling of lysine residues in the putative active site of Ad.5 DNA polymerase.

Differential labelling (Koshland et al, 1959) using PLP as an affinity label was the experimental approach taken to obtain more evidence on the structure of the active site of Ad.5 DNA polymerase. PLP was ideal to use as the imine bond (Schiff base) it can form with lysine residues may be radioactively labelled by reduction with tritiated $NaBH_4$ (see figure 9 in discussion). An outline of the ^{3}H differential labelling procedure is shown in figure 4.1.

4.2 Comparative tryptic mapping of PLP treated and substrate protected Ad.5 DNA polymerase.

Peptides from the trypsin digested ^{3}H affinity labelled Ad.5 DNA polymerase protein in the presence and absence of substrates were

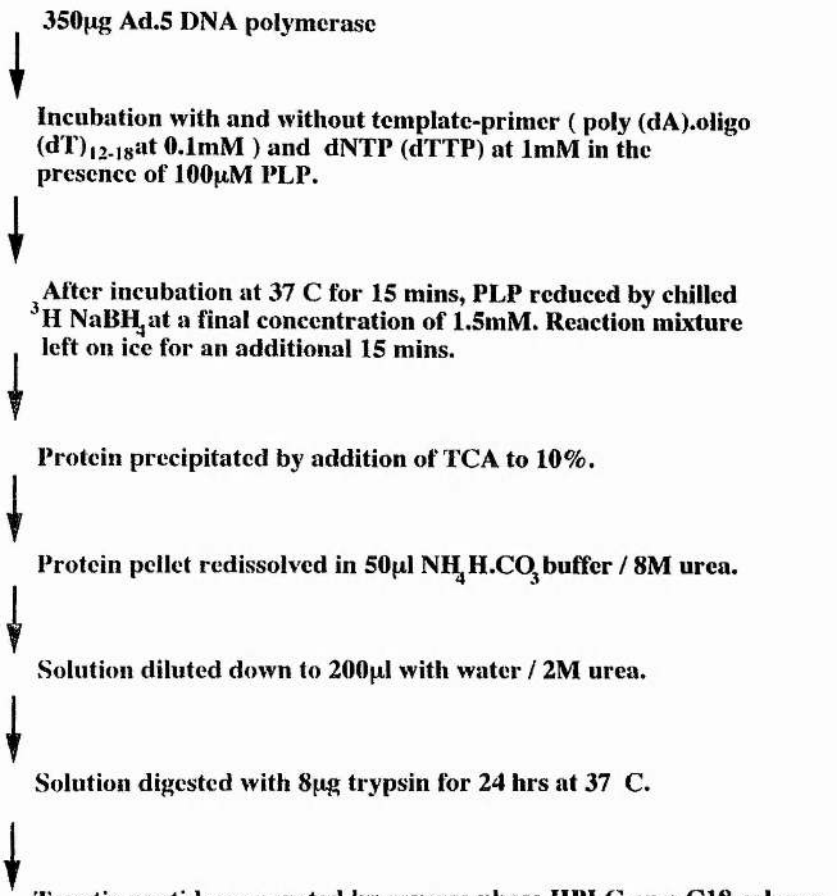


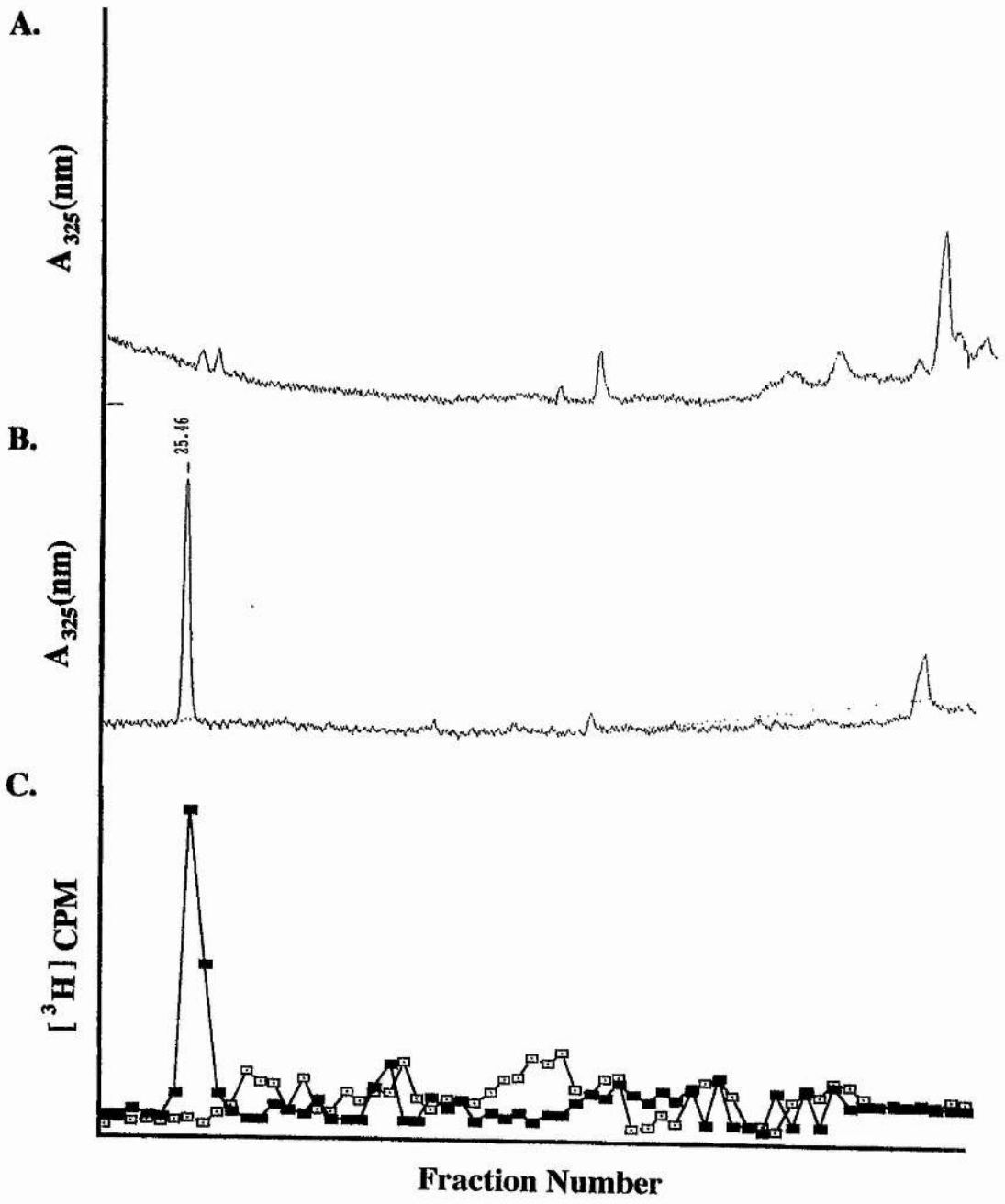
Figure 4.1 Outline of ^{3}H sodium borohydride labelling / substrate protection scheme for lysine residues in the active site of baculovirus purified Ad. 5 DNA polymerase.

Given above is the outline of the active site substrate protection scheme used to label 350µg (2.5 nmoles) of purified Ad.5 DNA polymerase protein.

fractionated by reverse phase HPLC chromatography using a Waters HPLC system with a Delta Pak C18 column , eluted from the column with 0-98% acetonitrile gradient (Materials and Methods) and collected over 105 x 0.5ml fractions. Eluted fractions were monitored at 325nm for reduced Schiff base characteristic absorbance (figure 4.2A, no templates and figure 4.2B, templates present) and10µl aliquots of each fraction dissolved in 5ml of Ecoscint A and scintillation counted. The plot of ^{3}H radioactivity (in the absence and presence of templates), versus fraction number (figure 4.2C) showed a relatively simple pattern of radioactive peptides in the HPLC eluate. A large peak of radioactivity was found between fraction numbers 5-10 in both cases, which most probably corresponds to unreacted tritiated $NaBH_4$ as this elution time corresponds to column flowthrough. Further comparison of the HPLC traces reveals that only one distinct peak of radioactivity can be seen, only in the absence of templates, between fraction numbers 23-27. This peak was subjected to N-terminal Edman degradation. Unfortunately it was not possible to identify a unique sequence with the isolated peptide.

Figure 4.2. Reverse-phase HPLC separation of tryptic peptides of Ad.5 DNA polymerase.

The tryptic peptides derived from the PLP modified enzyme were resolved on a Vydac C18 column and equilibrated with 0.06% trifluoroacetic acid. Peptides were eluted by increasing the concentration of solution B (98% acetonitrile containing 0.06% trifluoroacetic acid) as follows: 0-37% B (0-60 mins), 37-75% B (60-90 mins) and 75-98% (90-105 mins).The tryptic maps shown here correspond to, (A) Ad.5 DNA polymerase modified by PLP in the presence of dTTP and poly d (A). oligo d $(T)_{12\text{-}18}$, (B) Ad.5 DNA polymerase modified by PLP in the absence of template-primer and dNTP and (C) ^{3}H radioactivity of eluate fractions from both (A □—□) and (B ■—■) above.


A.
A325(nm)
B.
25.46
A325(nm)
C.
[3H] CPM
Fraction Number

DISCUSSION.

Adenoviruses replicate their genomes via a specialised "protein-priming" mechanism. In this system of DNA replication, it is believed that specific initiator protein(s) interact with the viral origin of replication, giving rise to the unwinding of the double-helix , leading to the creation of an exposed region of ssDNA which is used to elongate the rest of the genomic DNA. In adenovirus type 2 and 5 DNA replication, these proteins form a nucleoprotein complex consisting of five proteins: pTP, Ad.pol, Ad.DBP (all virally encoded), NF-I and NF-III (cellular proteins). The lack of any detectable helicase activity associated with any of the proteins (Lindenbaum et al., 1986) presents a problem when trying to understand the mechanism of DNA replication in the virus. The mechanism of DNA unwinding and its role in DNA replication has been studied in a large number of prokaryotic and eukaryotic reconstituted replication systems. This has led to the discovery of various DNA helicases that are essential for viral replication (Matson and Kaiser-Rogers, 1990 ; Matson et al., 1994). The results presented in chapter 2 attempt to clarify the process of DNA unwinding that takes place during the replication of the Adenovirus genome.

All the purified replication proteins present in the nucleoprotein complex were tested individually for DNA helicase activity (see results section chapter 2, Table 2.1). No detectable helicase activity was found in any of these proteins when tested at low concentrations in a standard in vitro DNA helicase assay. However, when different combinations of the proteins were tested at concentrations equivalent to those used in vitro initiation assays, DNA unwinding of substrate was detected. The activity was detected only when the viral protein Ad.DBP was present at concentrations which could completely saturate the DNA substrates.The

protein was termed as having helix-destabilising properties. In depth characterisation of this activity was carried out in results chapter 2.

1. Helix-destabilising property of the adenovirus DNA-binding protein.

The adenovirus DBP has been shown to possess the properties of a helix destabilising protein. When templates contain a large amount of single stranded DNA, the double stranded portion is efficiently unwound in a highly cooperative reaction. Completely double stranded templates are also unwound but this reaction is restricted by the length and G+C content of the DNA fragment. Like other helix destabilising proteins, DBP promoted unwinding requires neither ATP nor $MgCl_2$ (chapter 2, table 2.2). In fact, the latter is inhibitory to the process as are other agents, such as NaCl, which increase the stability of duplex DNA molecules (chapter 2, figure 2.5). Very similar properties have recently been ascribed to both the HeLa and herpes simplex virus coded single strand specific DNA binding proteins, which appear to be involved in cellular and viral DNA replication (Goergaki et al., 1993 ; Boehmer et al., 1993). Two possibilities therefore exist to explain the ability of DBP to unwind completely double stranded DNA. In one model DBP could utilise its ability to first bind to double stranded DNA (Cleat and Hay, 1989 ; Stuiver and van der Vliet, 1990), invade the DNA duplex and bind in a stable fashion to the exposed single strands. An alternative model is that DBP binds to transiently single stranded regions of DNA that are exposed during "breathing" of short double stranded DNA molecules. What is clear however is that when the double stranded DNA fragments are tightly bound by either NFI or NFIII at their cognate recognition sites DBP is unable to unwind the DNA. This could be due to the bound proteins stabilising the DNA duplex or alternatively the bound proteins

may interfere with the ability of DBP to form a continuos protein chain on the DNA. Unlike DNA helicases which translocate unidirectionally on DNA, there does not appear to be a strict directionality to the unwinding reaction catalysed by DBP. In the experiment designed to address this point (chapter, figure 2.6C) it was noted that the smaller fragment was released from the template at lower concentrations of DBP than the larger DNA fragment. While this is interpreted as being a consequence of the lower T_m of the smaller fragment, it cannot be ruled out that initial binding of a single molecule of DBP is followed by unidirectional polymerisation of the protein on the single stranded DNA. Determination of the three dimensional structure of DBP by X-ray crystallography (Tucker et al., 1993) has suggested a mechanism for the cooperative binding of DBP to single stranded DNA. It is thought that an extension at the C-terminus of one DBP molecule "hooks" into a complementary surface on an adjacent DBP molecule and in doing so initiates formation of a protein chain of DBP molecules. The ability of DBP to impose a rigid structure on DNA has recently been shown to be responsible for the ability of DBP to also promote renaturation of complementary single strands (Zijderveld et al., 1993). However, whereas the denaturation reaction described here is inhibited by high concentrations of monovalent and divalent cations, the renaturation reaction described above is highly resistant to their presence (Zijderveld et al., 1993).

The ability of DBP to destabilise double stranded DNA duplexes could be utilised at a number of points in the viral replicative cycle. While DBP has been shown to stimulate the initiation of Ad2 DNA replication in vitro by decreasing the Km for transfer of dCMP onto pTP and by increasing the binding of NFI to the replication origin, neither of these reactions are likely to involve the melting of duplex DNA.

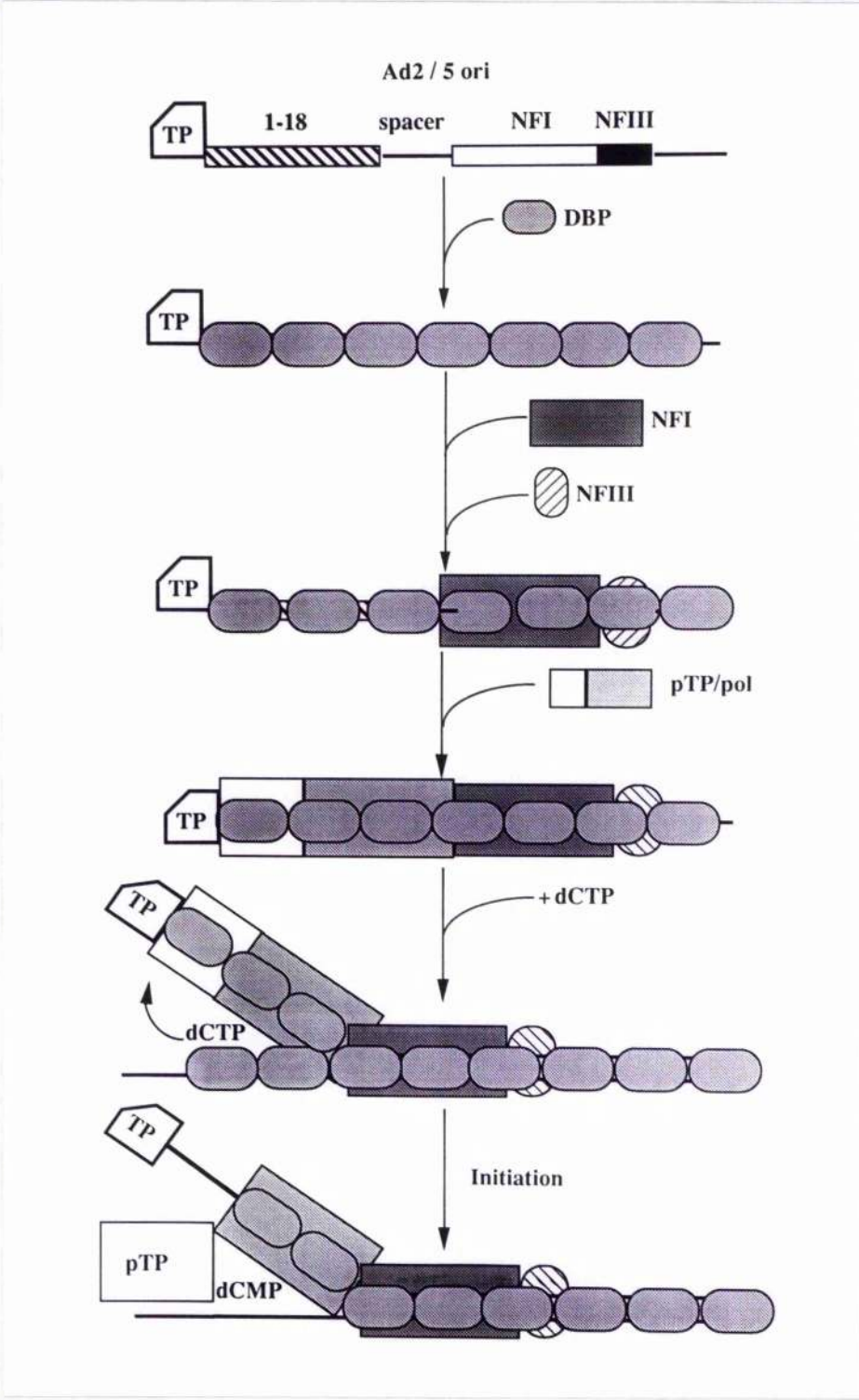


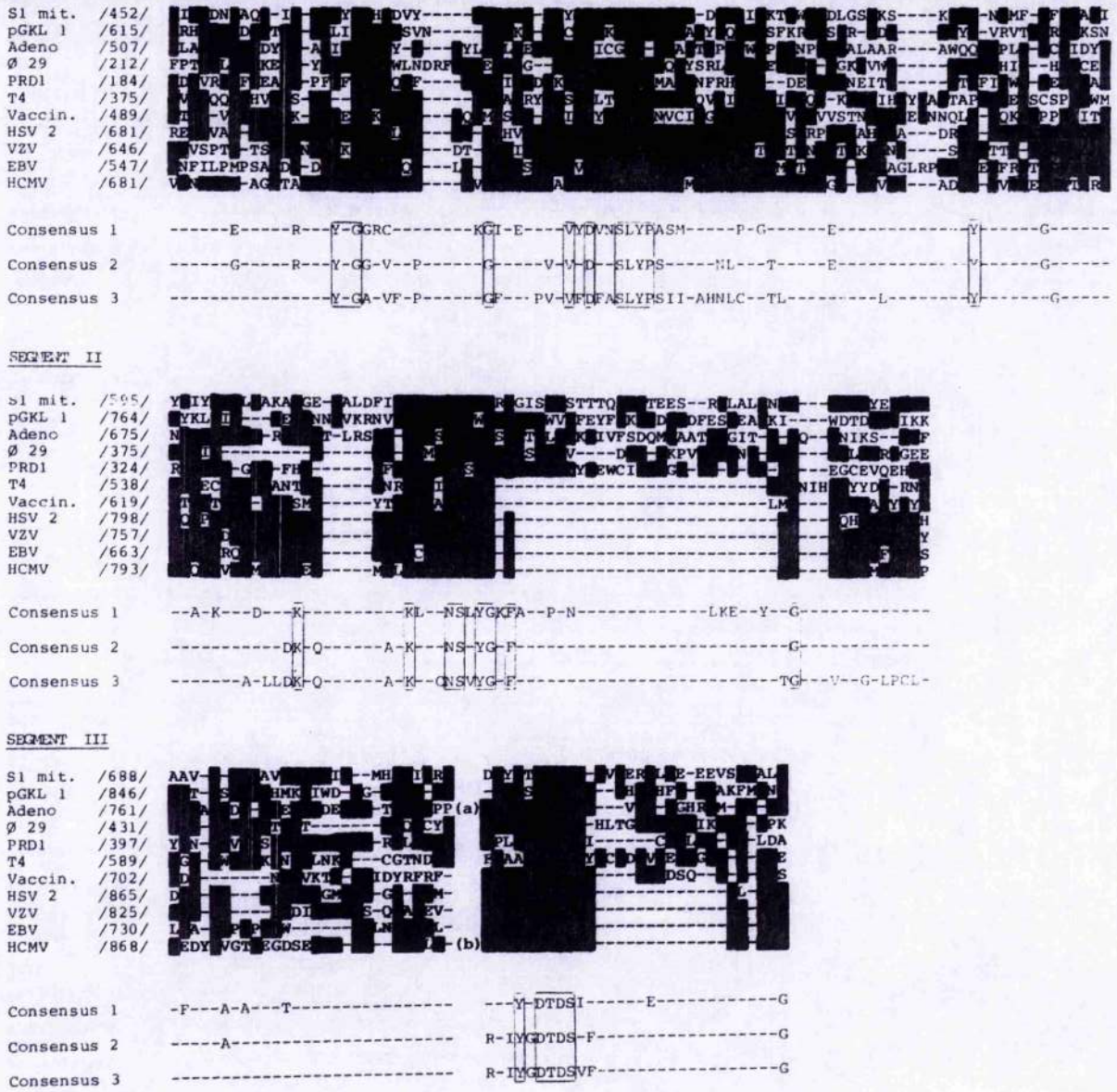
Figure 7. Strand opening and formation of a preinitiation complex at the adenovirus origin.Representations of known and potential DNA-protein and protein-protein interactions are shown.

However, DBP may stimulate the initiation of DNA replication by additional means and it has previously been demonstrated that Ad4 DBP dramatically stimulates initiation of DNA replication. In this case the extent of stimulation is independent of the concentrations of NFI and dCTP (Temperley and Hay, 1991). One possibility is that DBP may participate with other replication proteins in the unwinding of the DNA double helix that is expected to take place at the termini of the genome prior to initiation (chapter2, see figure 7). Numerous experiments have indicated that TP(Pronk et al., 1992, Pronk and van der Vliet, 1993), NF-I (De Vries et al., 1987 , Mul and van der Vliet, 1992) and NF-III (Verrijzer et al., 1991) can all distort adenovirus genomic DNA to some extent. Along with DBP's ability to distort and unwind short fully duplex DNA, this could provide a mechanism by which the adenovirus replication proteins can destabilise and eventually open the origin DNA. The requirement for DBP during progress of the replication fork has been well established and it is likely to be a consequence of DBP stabilising displaced single strands and altering the properties of the viral DNA polymerase. In the latter case DBP has been shown to convert pol into a form that is capable of strand displacement and highly processive DNA synthesis (Field et al., 1984 ; Lindenbaum et al., 1986). Both of these activities may well be a consequence of the ability of DBP to transiently destabilise double stranded DNA at the advancing replication fork.

2. PLP inhibition of the Ad.5 DNA polymerase.

Initial sequence homology studies on eukaryotic and prokaryotic DNA polymerases indicated the presence of clusters of highly conserved amino acids, posing the question as to whether the conservation was in any way a reflection of the functional importance of these regions to the polymerases. Thus far, two main groups of DNA polymerases have been

Figure 8. Homology segments among several DNA polymerases of prokaryotic and eukaryotic origin. Alignment was carried out as described in Bernad et al., (1987). Numbers at the left indicate the first residue in each sequence. A residue was considered consensus if it was present in more than half of the polypeptides considered. Consensus 1 indicates the consensus residues between DNA polymerases of terminal protein containing genomes. Consensus 2 indicates the consensus residues between all DNA polymerases. Consensus 3 indicates the consensus residues between the remaining DNA polymerases.


S1 mit. /452/
pGKL 1 /615/
Adeno /507/
Ø 29 /212/
PRD1 /184/
T4 /375/
Vaccin. /489/
HSV 2 /681/
VZV /646/
EBV /547/
HCMV /681/
Consensus 1
Consensus 2
Consensus 3
SEGMENT II
S1 mit. /595/
pGKL 1 /764/
Adeno /675/
Ø 29 /375/
PRD1 /324/
T4 /538/
Vaccin. /619/
HSV 2 /798/
VZV /757/
EBV /663/
HCMV /793/
Consensus 1
Consensus 2
Consensus 3
SEGMENT III
S1 mit. /688/
pGKL 1 /846/
Adeno /761/
Ø 29 /431/
PRD1 /397/
T4 /589/
Vaccin. /702/
HSV 2 /865/
VZV /825/
EBV /730/
HCMV /868/
(a)
(b)
Consensus 1
Consensus 2
Consensus 3

distinguished on the basis of their amino acid sequence relatedness: 1. E.coli Pol I and phage T7 DNA polymerase; 2. α-like DNA polymerases, which include both prokaryotic and eukaryotic, protein-primed and RNA-primed DNA replicases, probably sharing a common polymerisation mechanism. The adenovirus DNA polymerases are contained in the second group, the α-like DNA polymerases. Limited structural characterisation on the Ad.2 DNA polymerase (Field et al., 1984 ; Chen and Horwitz, 1989 ; Joung et al., 1991) and computer-generated analysis of related eukaryotic gene sequences (Larder et al., 1987a ; Wang et al., 1988) suggests that the catalytic domain of the Ad.5 DNA polymerase is likely to be located in the C-terminal region of the protein, between amino acid residues 687-726 (see figure 8). These amino acids correspond to a conserved region of sequence homology (Region III) which contains the consensus sequence "K _ _ _ N _ _ YG", found between different viral DNA polymerases (Larder et al., 1987a). This evidence suggested that a lysine residue (K) could be important for catalytic activity.

Chemical modification agents can be used successfully to identify amino acid residues that are important for enzyme activity, provided that the following two criteria are fulfilled: firstly, chemical modification of the residues must result in a loss of enzyme activity and secondly, this inactivation must be prevented by substrates or substrate analogues. Pyridoxal-5'-phosphate (PLP) is an ideal chemical modifier in this respect. The sensitivity of a number of prokaryotic (Modak, 1976 ; Basu and Modak, 1987), eukaryotic DNA polymerases (Diffley, 1988) to PLP prompted its use in this study. Inhibition kinetics with these different DNA polymerases indicated that it was a specific, substrate dNTP-binding site-directed reagent, that could be incorporated covalently into an enzyme's active site via borohydride reduction of the PLP-enzyme

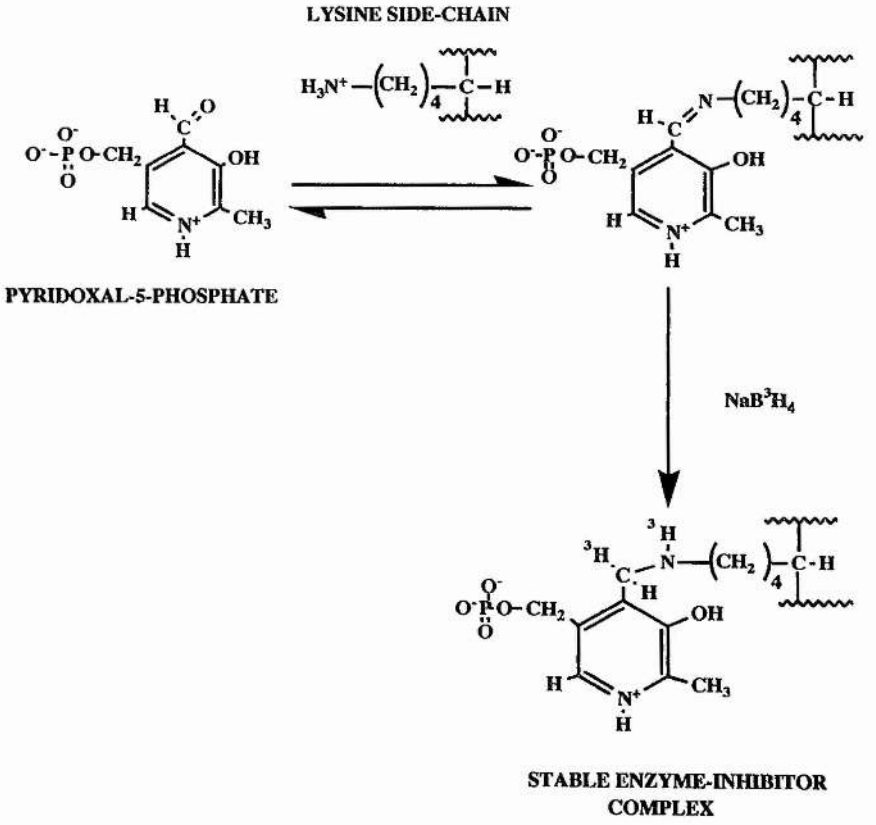


Figure 9. PLP inhibition reaction pathway. The formation of the reversible imine bond with a lysine residue and its reduction to a secondary amine by sodium borohydride (radioactive) are shown.

complex formed during inhibition. PLP specifically modifies the amino acids, arginine and lysine. In all of the studies on the DNA polymerases mentioned above, a lysine residue in or near the active-site has been implicated as the residue involved in PLP mediated inhibition. The data presented in chapter 3 of this study characterises the PLP inhibition of Adenovirus type 5 DNA polymerase and lays down the groundwork for the use of this inhibitor in the affinity labelling of the catalytic DNA polymerase's active-site.

PLP inhibition of the Ad.5 DNA polymerase occurs via the formation of a metastable imine ("Schiff base") with an amino acid group (either arginine or lysine) on the enzyme. Nearly all of the polymerases inhibition can be reversed with primary amine (Tris-HCl) or substrate, indicating that the inhibition by PLP occurs via reversible imine bond formation. The rate of PLP inhibition of the polymerase, in the presence and absence of magnesium ions, does not increase linearly with increasing PLP concentration (chapter 3, figure 3.2). The rate reaches a plateau at high concentrations of PLP, exhibiting saturation kinetics. This argues that PLP is a specific inhibitor of the polymerase, binding to a specific site on the enzyme in a non-covalent fashion prior to the formation of a covalent bond, in this case a reversible imine (see figure 9). This is an important property of an affinity label. The rate of PLP inhibition is increased two-fold in the presence of magnesium ions, indicating that PLP probably chelates with the Mg^{2+} ions and forms a more potent inhibitor for the polymerase. A magnesium ion chelate of PLP has been shown to be required for effective inhibition of the E.coli DNA polymerase I (Basu and Modak, 1987) and the murine leukemia virus reverse transcriptase (Basu et al, 1988).

The substrate protection data presented in chapter 3, along with the known order of substrate binding in related eukaryotic DNA

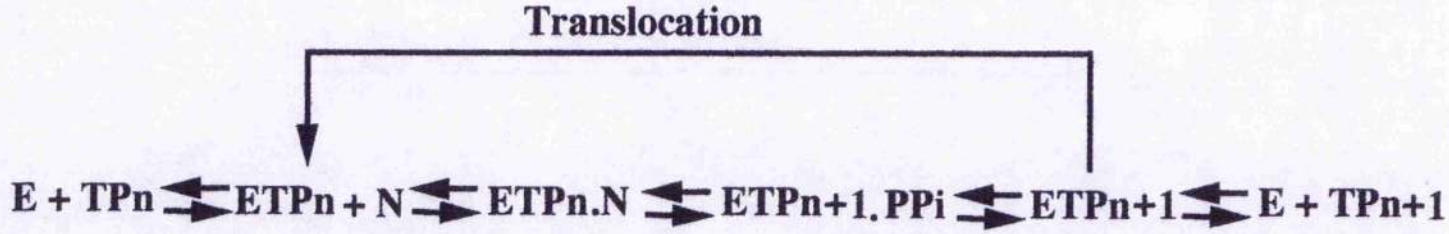
polymerases, such as DNA polymerase α from Drosophilia, suggests that PLP can inhibit Ad.5 DNA polymerase by binding to one site on the enzyme. Protection from this inhibition requires the presence of a dNTP and an appropriate template-primer, e.g. the presence of dTTP, will provide protection for Ad.5 DNA polymerase from PLP inhibition only when poly d(A).oligo d(T) is present (chapter 3, table 3.1). This data strongly suggests that the process of substrate binding in Ad.5 DNA polymerase is dependent on the prior binding of template-primer and that only dNTP which is complementary to the template strand can bind and be accepted by the active-site of the polymerase. Alternatively, the reversal of PLP mediated inactivation by the inclusion of template-primer and substrate dNTP may be argued to result from another PLP reactive site, which may be required for the translocation of the enzyme during polymerisation of dNTP's. If this catalytic site was modified, one may still expect perfect substrate binding although turnover of bound substrate may be affected. In E.coli DNA polymerase I and DNA polymerase α from Drosophilia melanogster, PLP-mediated inactivation has indeed been shown to occur via the reactivity of at least 2 lysine residues, one involved in substrate binding and the other in catalysis (Basu and Modak, 1987 ; Basu et al., 1988 ; Diffley, 1988). However, this idea isn't consistent with the kinetic data in chapter 3 and the peptide mapping studies in chapter 4, which indicated that only one peptide containing PLP was present after digestion with trypsin (see chapter 4).

Initial velocity experiments demonstrate that PLP inhibition of the polymerase is not competitive with any substrate (chapter 3, figure3.5). Since essentially all the polymerase can be protected by the formation of the primer-template/dNTP ternary complex, these results indicate that PLP inhibition must occur at some point after the formation of this ternary complex in one polymerisation cycle and before the formation of

a ternary complex in the next cycle. This is illustrated in figure10. The results in chapter 3 argue that both the polymerase species, E and ETPn, can be inhibited by PLP binding, while the polymerase ternary complex species ETPn.N is protected from PLP inhibition. This in turn indicates that either the catalytic step, pyrophosphate release and/or translocation of the newly formed primer-binding site must be the rate-limiting step in the polymerisation cycle. During this rate-limiting step the dNTP-binding site would be accessible for PLP binding and inhibition. Alternatively, the 3'-5' exonuclease associated with the adenovirus DNA polymerase could have its proof-reading (implicated) function exercised after catalysis and/or during translocation, making it the rate-limiting step predicted from PLP inhibition.

The associated 3'-5' exonuclease activity, which co-sediments with the DNA polymerase , exhibits negligible sensitivity to PLP (chapter 3, figure 3.7). This implies that there are two distinct active-site domains for the catalysis of these two activities. This is supported to some degree by sequence homology comparisons (see figure11) on a wide range of prokaryotic and eukaryotic (including Ad.2 DNA polymerase) DNA polymerases (Bernad et al., 1989). The conclusion from these studies was that the amino acids used for some of the different enzymatic activities required to catalyse the synthesis of DNA had been assembled in the same polypeptide to form a mutlifunctional DNA polymerase. If this hypothesis is correct, the organisation of the DNA polymerases into modular structures would imply a relative functional and structural independence of each catalytic domain. The results in chapter 3 argue that, functionally, this is the case with the adenovirus type 5 DNA polymerase. However, no x-ray crystal structure for any of the adenovirus DNA polymerases exists at the moment, so definitive studies cannot be carried out.

Figure 10. The polymerisation cycle of Ad.5 DNA polymerase. The substrates, transition complexes and products are as indicated.



E = Ad.5 DNA Polymerase

Substrates:

TPn = Template-primer

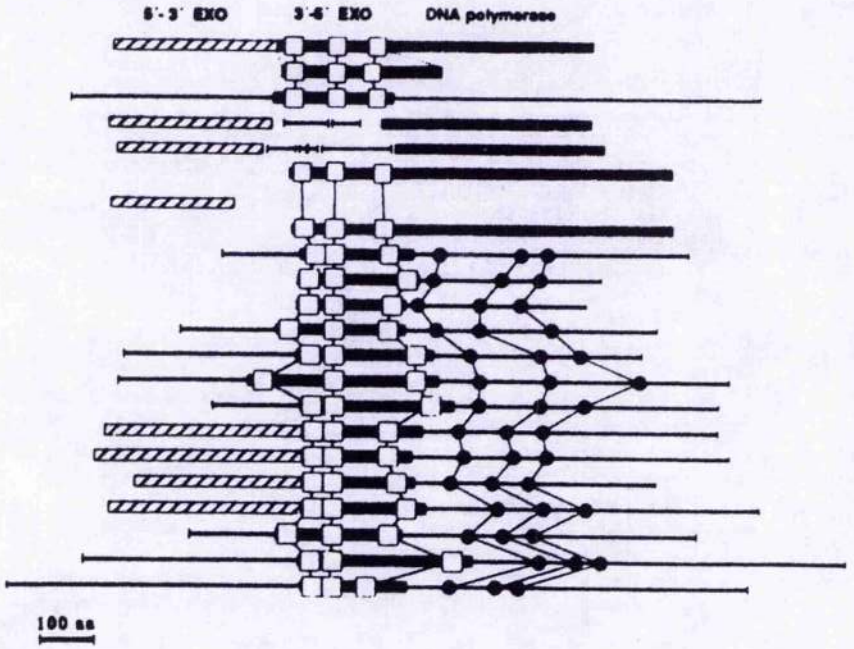
N = dNTP

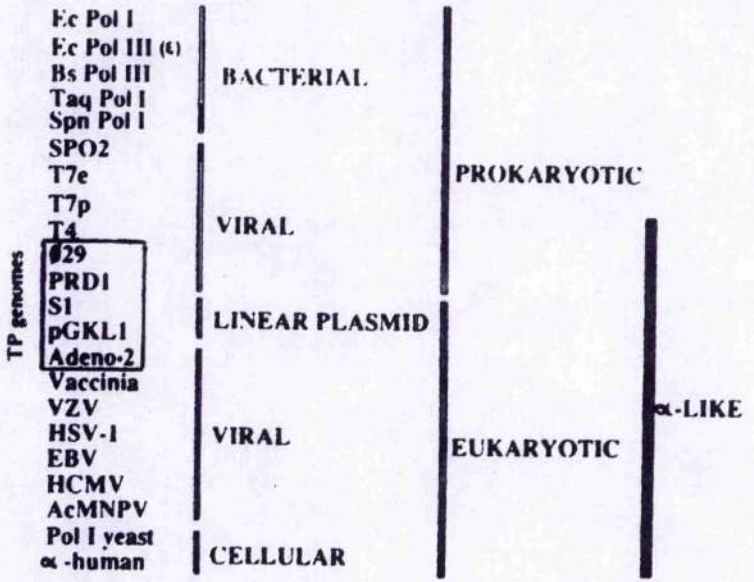
Products:

TPn+1 = Template-primer elongated by one nucleotide

PPi = Inorganic pyrophosphate

Figure 11. Relative arrangement of homologous regions among prokaryotic and eukaryotic DNA polymerases.Each DNA polymerase or exonuclease subunit is represented with the N terminus at the left hand side. Cross-hatched and thick-blackened regions indicate homology with the 5'-3' exonuclease, 3'-5' exonuclease and polymerase domains, respectively, of E.coli Pol I. Undetected or nonsignificant homology is represented by a straight line. Open boxes represent N-terminal homologous segments within the DNA polymerases and are proposed to be a general 3'-5' exonuclease active site. Filled circles indicate the homologous C-terminal segments present in α-like DNA polymerases (Larder, 1987).


5'-3' EXO
3'-5' EXO
DNA polymerase
100 aa


Ec Pol I
Ec Pol III (α)
Bs Pol III
Taq Pol I
Spn Pol I
SPO2
T7e
T7p
T4
ϕ29
PRD1
SI
pGKL1
Adeno-2
Vaccinia
VZV
HSV-1
EBV
HCMV
AcMNPV
Pol I yeast
α-human
TP genomes
BACTERIAL
VIRAL
LINEAR PLASMID
VIRAL
CELLULAR
PROKARYOTIC
EUKARYOTIC
α-LIKE

To date, only in the case of E.coli DNA polymerase I have the different activities present in a DNA polymerase been ascribed to physically separate domains. Based on crystal analysis of its Klenow fragment (C-terminal region of the protein that contains both the DNA polymerase and 3'-5' exonuclease activities) with DNA, it has been proposed that one of the critical features for its role in 'editing" Okazaki fragments is that the polymerase and exonuclease active sites are separated, and are either located on a different subunit, as in the case of E.coli DNA pol III HE (Kong et al., 1992), or on a different structural domain, as in the case of E.coli DNA polymerase I (Ollis et al., 1985).

The last result in chapter 3 indicates that PLP inhibition of the Ad.5 DNA polymerase can influence its function in the initiation of adenovirus DNA replication. The polymerase is thought to initiate replication (pTP-dCMP complex formation) by directly catalysing the formation of a phosphodiester bond between the β-OH group of a serine residue in the preterminal protein (Lichy et al., 1982). Inhibition of Ad.5 DNA polymerase by PLP, prior to its incubation in an in vitro initiation assay, resulted in a reduction of the pTP-dCMP complex signal. PLP inhibition of pTP had no effect on its initiation function. This argues that the initiation and polymerisation functions may share the same active-site. However, no mutational studies have been carried out on the conserved region III (putative dNTP binding site) of the polymerase to substantiate the above claim. Analysis has been carried out on conserved region I (putative metal-binding site) of the polymerase. Mutations in this region affected specific initiation and elongation reactions on different adenovirus templates (Joung et al., 1991). These results imply that region I is important in the process of dCMP transfer during initiation. However, more extensive studies on the related protein-priming φ29 DNA polymerase has revealed that its four most conserved regions are all

critical in the initiation of its DNA replication process (Bernad et al., 1990 ; Blasco et al., 1993). A site-directed mutagenesis approach has been used to describe the functional significance of all the conserved regions characterised by the motifs "Kx_3NSxYG", "YGDTDS", "KxY" and "Dx_2SLYP"; all the mutant polymerases from these conserved regions showed normal 3' to 5' exonuclease activity but were affected in their synthetic activities (protein-primed initiation and/or DNA polymerisation). Motif "Kx_3NSxYG" has been proposed to be involved in template-primer binding and dNTP selection (Blasco et al., 1992b, 1993) ; ϕ 29 DNA polymerase residues Asp^{456} and Asp^{458} of conserved motif "YGDTDS" have been proposed to be involved in metal binding and catalysis (Bernad et al., 1990), and mutations in conserved motif "KxY" have been shown to affect primer recognition (Blasco et al., 1992a). In addition, a mutant in residue Tyr^{254} of ϕ 29 DNA polymerase conserved motif "Dx_2SLYP" has been described to be affected in Me^{2+}-dNTP binding (Blasco et al., 1992b). Recently more extensive mutagenesis studies have been carried out on this conserved region (Blasco et al., 1993). Their results suggest that residue Asp^{249} in the ϕ 29 DNA polymerase could have a direct role in catalysis. They have proposed that Asp^{249} , together with Asp^{456} and Asp^{458} , may be close to each other in the three-dimensional structure of ϕ 29 DNA polymerase, possibly contributing to catalysis by acting as metal ligands. Similar groups of acidic residues have been described to be critical for the maintenance of the synthetic activities in E.coli DNA polymerase I Klenow fragment (Polesky et al., 1990, 1992) and HIV-I reverse transcriptase (Larder et al., 1987b, 1989). More extensive mutational analysis needs to be carried out on all of the conserved regions in Ad.5 DNA polymerase in an attempt to clarify the amino acid residues important in both initiation and elongation of DNA replication.

3. Identification of amino acid residues in the active site of the Ad.5 DNA polymerase.

On the basis of comparative tryptic peptide mapping of Ad.5 DNA polymerase modified by PLP, in the presence and absence of template-primer and substrate dNTP, a single putative pyridoxylated peptide was identified. The peak was found only in the absence of substrates. The peak was identified as being pyridoxylated on the basis of: A. its absorbance pattern at 325nm (reduced "Schiff base" characteristic absorbance) and B. the distribution of radioactively reduced sodium borohydride in the tryptic digest. However, amino acid composition and sequencing analyses revealed that the peak contained no detectable amounts of peptide. The lack of sequence data could be attributed to several things. The most likely of these is that not enough protein may have been used in the initial inhibition reaction with PLP. The inhibition reaction is followed by precipitation by trichloroacetic acid (TCA) and then HPLC on a C-18 peptide column. The combination of these two processes may have reduced the amount of PLP inhibited polymerase available for sequencing. Strong evidence for this comes from the fact that the amount of labelled material in the HPLC purified peptide peak is 10-fold lower than the amount of protein initially added into the reaction. An alternative explanation is that the reduction of PLP by the radioactive sodium borohydride may simply not have worked. However, the former seems to be the case, as samples taken from the PLP inhibition reactions before TCA precipitation were found to be modified (no substrates) and unmodified (substrates present) when tested in a standard in vitro DNA polymerase activity assay.

In conclusion, the data presented in chapters 3 and 4 demonstrate that the polymerase activity of Ad.5 DNA polymerase can be inhibited by

PLP. Although direct physical evidence for the involvement of lysine 695 in this process was not found, it seems likely that with larger quantities of the protein becoming available, an x-ray crystal structure for the protein will become a reality and pinpoint with accuracy the role of a lysine residue in catalysis.

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